REGULATION OF EPITHELIAL MORPHOGENESIS BY *DROSOPHILA* RHO GTPASE AND ABL KINASE

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ABSTRACT

Donald T. Fox: Regulation of epithelial morphogenesis
by *Drosophila* Rho GTPase and Abl kinase
(Under the direction of Dr. Mark Peifer)

The goal of embryogenesis is to convert a fertilized egg into an adult. To ensure that this process occurs normally, regulatory proteins act on the structural elements of a cell to change its position or shape. The resulting changes at a cellular level are coordinated within tissues and constitute a process termed morphogenesis. To date, many morphogenesis regulators have been identified in many model systems. The current aim is to understand how each regulator interacts with the others and with the structural elements of the cell such as the cell adhesion machinery and the cytoskeleton. In my thesis work, I have utilized epithelial development in *Drosophila* embryos as a model to investigate functions of the morphogenesis regulators Rho1 GTPase and Abelson (Abl) kinase.

Rho GTPases have been linked to both regulation of cell-cell adhesion at adherens junctions (AJs) and the actin cytoskeleton. With respect to adhesion, conflicting evidence exists concerning how Rho interacts with core AJ components and the accessory AJ protein p120-catenin (p120). As part of my thesis work, I examined the role of *Drosophila* Rho1 during epithelial morphogenesis. I found that Rho1 function is not dependent on p120, but that Rho1 regulates core AJ components. Further, my work suggests a mechanistic role for Rho1 in trafficking of the AJ protein cadherin.

The second major area of my work examined the role of the non-receptor tyrosine kinase Abl during *Drosophila* morphogenesis. I identified a novel role for Abl in a specific

type of apical cell shape change. Abl is required for apical constriction of mesodermal cells during gastrulation. Abl's regulation of its target Enabled and, consequently, the apical actin cytoskeleton is crucial for this function. This observation led me to test the roles of other ventral furrow regulators in apical actin regulation. I found that RhoGEF2 but not Concertina (G-alpha 12/13) also regulates apical actin during ventral furrow formation, possibly clarifying the difference in phenotypes of mutants of these two morphogenesis regulators. Finally, I developed and characterized new tools for studying Abl localization and activation. Overall, these studies will aid in efforts in understanding how to build an animal.

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CHAPTER 1

HOW TO BUILD AN ANIMAL: THE REGULATION OF MORPHOGENESIS

From the point of view of a developmental biologist, the most exciting time of your life occurs before you are even born. From fertilization to birth, embryonic development involves the dynamic forming and rearranging of the tissues that will make up the adult body plan. The question of how an animal is built is the fundamental question of developmental biology. To understand this process at a molecular level, one must consider the interactions between the various parts that shape and reshape an embryo. Because the unit of tissue structure is the cell, we must consider what gives a cell its shape. Further, we need to understand how a cell associates with its neighbors to undergo coordinated rearrangements in tissue structure. Finally, we must identify and characterize the roles of signaling proteins that instruct a cell to alter its shape or position. In this era of largely annotated genome sequences, what developmental biologists have to work with is a "parts list" for the assembly of an animal. The daunting but achievable task now is to figure out how the parts work together to mold the embryo into the adult structure.

In the Peifer lab, we have approached this problem by observing *Drosophila* embryogenesis. In addition to the wealth of genetic tools in this system, the *Drosophila* embryo provides an excellent model to observe the role of candidate morphogenesis regulators. During embryogenesis, a number of distinct cell shape changes occur at specific times and locations. These morphogenetic events closely mirror processes that occur in

mammals, such as apical cell constriction. As the term morphogenesis encompasses a myriad of processes in many tissue types, we have limited our focus to those involving cells of the epidermis. At a molecular level, we have focused our attention on proteins that act in the apical domain of cells. At this subcellular location, a hub of protein activity resides at the cell-cell adhesion complex known as the adherens junction. Adherens junctions are closely coupled to the actin cytoskeleton and are the location of many signaling proteins. Thus, understanding the interaction between adherens junctions, the cytoskeleton, and signaling proteins during fly embryogenesis can serve as a microcosmic model of morphogenesis.

During my time in the Peifer lab, I have focused on the role of two signaling proteins with ties to adherens junction and cytoskeletal regulation. I evaluated the role of the small GTPase Rho1 as an adherens junction regulator in *Drosophila* and determined whether, as in cultured mammalian cells, *Drosophila* Rho1 is regulated by the adherens junction protein p120-catenin (p120). Additionally, I examined the role of the non-receptor tyrosine kinase Abelson (Abl) during *Drosophila* morphogenesis. As part of this analysis, I characterized Abl's localization and activation. I also contributed to work concerning the role of p120 at adherens junctions and the function of Abl prior to morphogenesis.

The nuts and bolts

The first components to consider when building an animal are structural proteins. These proteins dictate cell shape and help to organize cells within tissues. The two main classes of these "nuts and bolts" parts are cell adhesion complexes and the cytoskeleton. These structural elements are often functionally linked to one another, as the actin cytoskeleton is to adherens junctions (reviewed in Gates and Peifer, 2005). Cell adhesion

and cytoskeletal proteins can hardly be thought of as rigid structures. Rather, both are quite dynamic and undergo turnover and rearrangements to allow for cell shape change and migration. In my thesis work, I focused on cell adhesion at adherens junctions and the interaction of this complex with the actin cytoskeleton.

Cell adhesion at adherens junctions

The adherens junction is a critical cell-cell adhesion complex in epithelial cells. This highly conserved complex consists of three core components: cadherin and two catenin proteins, alpha and beta (Fig. 1). Cell-cell contact is mediated by the extracellular domain of Cadherin, which binds calcium and mediates homophilic contacts with extracellular domains of cadherins of a neighboring cell. Within the cell, the cytoplasmic tail of cadherin binds to beta-catenin. Beta-catenin also binds alpha-catenin, which can bind actin (reviewed in Gumbiner, 1996). While this cadherin-catenin complex has been thought to bind to actin, recent evidence suggests that alpha-catenin can bind to beta-catenin or to actin, but not both at once (Drees et al, 2005; Yamada et al, 2005). Nevertheless, cell-cell adhesion at adherens junctions certainly influences the activity of the actin cytoskeleton.

Another component of adherens junctions is the catenin p120. Originally identified as a substrate of Src kinase, p120 binds to the cadherin tail proximal to the site of beta-catenin binding (Fig. 1, reviewed in Anastasiadis and Reynolds, 2000). While overexpression studies in cultured mammalian cells originally described both positive and negative roles for p120 in cell adhesion, siRNA knockdown studies overwhelmingly argue for a positive role of p120 in strengthening adhesion at adherens junctions (Davis et al, 2003; Xiao et al, 2003). Outside of junctions, mammalian p120 has been implicated in other cellular functions. Cytoplasmic p120 has been connected to the negative regulation of Rho

GTPase, while nuclear p120 seems to play a role in transcription (reviewed in Anastasiadis and Reynolds, 2000).

Contrary to the name, cell adhesion is not cell glue. Rather, cell adhesion is frequently remodeled to accomplish many morphogenetic events. For example, adherens junctions can expand to change the circumference of a cell, as during zebrafish epiboly (Kane et al, 2005) or expansion of the *Drosophila* trachea (Beitel and Krasnow, 2000). Decreased cell adhesion can also contribute to morphogenesis. Down-regulation of cadherin in a group of cells can create differential cell adhesion between those cells and the rest of a tissue. Such alterations in cell adhesion may allow for sorting of that group of cells out of the tissue, as during epithelial to mesenchymal transitions (reviewed in D'Souza-Schorey, 2005). A third way in which dynamic cell adhesion can contribute to morphogenesis occurs during cell migration. In both gastrulating *Xenopus* embryos and migrating border cells of *Drosophila* ovaries, the turnover of adhesive contacts between migrating cells and their substrate allows for making and breaking of adhesive bonds which drive cell migration (Geisbrecht and Montell, 2002).

Cell adhesion can also indirectly influence morphogenesis by acting as an upstream signal for cytoskeletal remodeling. Given its association with the actin cytoskeleton, it is not surprising that adherens junctions are implicated in actin regulation. Alpha-catenin appears to functionally interact with both formins (Kobielak et al, 2004) and Ena/VASP proteins (Vasioukhin et al., 2000), which regulate actin polymerization at adherens junctions. Further, cadherin-cadherin ligation has been shown to stimulate the activity of Rho family GTPases, known for their roles in actin reorganization (reviewed in Yap and Kovacs, 2003).

The Actin Cytoskeleton

While cell adhesion coordinates changes within a tissue, the force behind cell shape change is driven by the actin cytoskeleton. Polymerized filaments of actin are assembled by the addition of monomers into the growing or barbed end of an existing filament. The formation of new filaments is accomplished by nucleating proteins such as the Arp 2/3 complex or formins. The active assembly and disassembly of actin filaments is critical during cell migration (reviewed in Pollard and Borisy, 2003).

Polymerized F-actin filaments are not simply linear in structure but tend to be branched. Work in cultured mammalian cells has identified a mechanism for regulating the degree of actin branching. This work revealed that competition for barbed ends occurs between the Ena/VASP family of proteins, which promote the growth of an existing filament, and capping proteins, which block the further addition of monomers to one filament, thus forcing branched nucleation of new filaments (Bear et al, 2002). At the cellular level, branched and unbranched structures form cell protrusions called lamellipodia and filopodia that are key in cell migration (reviewed in Reinhard et al., 2001).

Another way to regulate actin structure is through contraction of filaments. Myosin II is the major motor involved in contraction of actin. Myosin II consists of a hexamer of two heavy, two essential light, and two regulatory light chains. In an ATP-dependent process, myosin slides actin filaments together (reviewed in Craig and Woodhead, 2006). Actin-myosin contraction contributes to a number of morphogenetic processes, such as the apical constriction that invaginates cells of the vertebrate neural tube or the mesoderm of the fly embryo. Such contraction also plays a conserved role during both *Drosophila* dorsal closure and zebrafish epiboly (Koppen et al., 2006), where sheets of epithelial cells are pulled together.

Rearranging the pieces

Embryonic morphogenesis encompasses many events, most of which likely involve cell adhesion and the cytoskeleton. The question then is how a group of cells in a tissue executes one morphogenetic change versus another. One hypothesis is that the nuts and bolts pieces are rearranged by a set of regulatory proteins specific to one process. For adherens junctions, these players are the many signaling proteins that interact with this complex. Two examples of these regulatory proteins are the small GTPase Rho and the non-receptor tyrosine kinase Abl.

Rho GTPase

Rho small (not heterotrimeric) GTPases are often described as "molecular switches." These proteins cycle between GDP-bound "off" and GTP-bound "on" states. In the on state, the active GTPase interacts with an effector protein. The Rho family of GTPases consists of Rho, Rac, and Cdc42 proteins. Classic studies in mammalian fibroblasts implicated each type of Rho GTPase in a different aspect of actin structure regulation. While Rho promotes actin stress fiber formation, Rac contributes to lamellipodial actin and Cdc42 to filopodial actin formation (Ridley and Hall, 1992, Ridley et al, 1992, Nobes and Hall, 1995. Since these studies, Rho family GTPases have been implicated in almost every cellular process, from mitosis to polarity to transcription. Specificity of Rho GTPase function is conferred by the activity of numerous Rho regulatory proteins. GEF proteins promote the active Rho state by exchanging GDP for GTP. Conversely, GAP and GDI proteins promote the inactive state by promoting GTP hydrolysis and sequestering GDP-bound Rho proteins, respectively (reviewed in Moon and Zheng, 2003).

While the effects of Rho on actin structure is clear, Rho's interaction with cell adhesion at adherens junctions is not as well understood. *Drosophila* Rho1 has been shown to bind directly to both alpha-catenin and p120 (Magie et al., 2002). Further, Rho appears to contribute to some of the same morphogenetic processes that involve adherens junction regulation, such as dorsal closure in *Drosophila* (Bloor and Kiehart, 2002). However, the exact relationship between Rho and adherens junctions is unclear, as mis-expression studies in both flies and cultured cells suggest both positive and negative roles for Rho in adherens junction regulation (reviewed in Perez-Moreno et al., 2003). As part of my thesis work, I used loss of function, rather than mis-expression analysis, to determine whether *Drosophila* Rho1 is an adherens junction regulator. Further, I tested the hypothesis that, as in mammalian cells, p120 regulates Rho1 function.

Abl Kinase

Another candidate piece mover in morphogenesis is the non-receptor tyrosine kinase Abl. Like Rho GTPases, Abl also exists in active and inactive states. Non-receptor tyrosine kinases may share a conserved mechanism of activation, as comparison of the crystal structures of Src and Abl kinases suggests. These proteins adopt a folded confirmation in the inactive state which obscures protein interaction domains and activating residues. A series of activation events then unfolds the kinase, allowing it to interact with effector proteins (reviewed in Harrison, 2003).

Tyrosine phosphorylation plays a major role in adhesion at adherens junctions. Such phosphorylation is thought to alter the ability of junctions to assemble, and mis-regulated adhesion is implicated as a step to cancer invasiveness (reviewed in Gumbiner, 2005). Studies in cultured mammalian cells have linked non-receptor tyrosine kinase signaling to

regulation of cadherin-mediated adhesion (reviewed in Gumbiner, 2005). In *Drosophila,* Abl has been implicated in the regulation of adherens junctions. *Drosophila abl* genetically interacts with *shotgun (E-cadherin)* mutants and regulates the localization of the single Ena/VASP homologue, which co-localizes with adherens junctions (Grevengoed et al., 2003; Grevengoed et al., 2001). Abl loss of function in *Drosophila* also disrupts morphogenetic processes that require adherens junction function, such as dorsal closure and head involution.

While the above evidence connects Abl to regulation of adhesion, Abl may also act more directly on the actin cytoskeleton. Unlike its close relative Src, Abl family kinases contain a long C-terminal region that can bind to both filamentous and monomeric actin. Mammalian Abl-related gene (Arg) can bundle actin filaments in vitro, and expression of Abl or Arg in cultured mammalian cells causes rearrangement of the actin cytoskeleton (reviewed in Hernandez et al., 2004). Loss of Abl in both fly and mouse embryos disrupts a number of morphogenetic processes, which coincide with a change in actin localization (Grevengoed et al., 2001; Koleske et al., 1998). Among the many Abl substrates are the Ena/VASP proteins. In early *Drosophila* embryos, loss of Abl results in ectopic Ena localization, resulting in ectopic apical actin-rich microvilli at the expense of basal actin in membrane furrows. This phenotype is rescued by reduction in the dose of Ena (Grevengoed et al., 2003).

While the above work ties Abl function to morphogenesis and actin regulation, it is not clear when and where Abl activity is needed and, of equal importance, not needed. In my thesis work, I have examined Abl's role in *Drosophila* morphogenesis in detail. As part of this analysis, I developed and made use of new Abl localization tools to observe where and when total and active Abl protein localizes. Additionally, I have initiated studies on identifying potential upstream activators of fly Abl, following up on the observation from

cultured mammalian cells that Abl is activated by receptor tyrosine kinases and Src (reviewed in Hernandez et al., 2004).

Epithelial morphogenesis in the *Drosophila* embryo: a quick tour

The *Drosophila* embryo provides an excellent model for observing morphogenetic events. The role of the "nuts and bolts" players has been examined in many of these processes. Embryogenesis lasts 24 hours and can be broken up into several distinct stages. The embryo begins as a syncytium during the blastoderm (Fig. 2A). A group of nuclei in this early embryo migrate to the surface and continue to divide. When this population reaches approximately 6,000 nuclei, each nucleus is simultaneously enclosed in a single membrane in a process known as cellularization (time to this point- 3 hours @ 25°C). Disruption of the actin cytoskeleton can inhibit cellularization (Planques et al., 1991). However, to this point, adherens junctions are dispensable for development (Cox et al., 1996; Grevengoed et al 2003). Thus, epithelial morphogenesis truly initiates after cellularization with the onset of gastrulation.

Gastrulation involves the internalization of the mesoderm and posterior midgut cells (Fig. 2B, arrow) as well as the elongation of the embryo in the A-P axis, a process referred to as germband extension (Fig. 2B,C). Cell shape changes during gastrulation consist of apical constrictions in the mesoderm and posterior midgut, as well as cell-cell intercalation in the germband. Apical myosin II localization appears key for apical constriction during gastrulation and other such cell shape changes in the embryo (Nikolaidou et al 2004). Further, adherens junctions are thought to act as a link to the contractile myosin network during constriction (Dawes-Hoang et al., 2005). Adherens junctions also rearrange cell-cell

contacts to allow cell intercalation in the extending germband (Bertet et al., 2004). During germband extension, segmentation of the embryo begins to be apparent (Fig. 2C, time to fully extended germband- 9 hours @ 25°C).

During germband extension a new cell type emerges on the surface of the embryo known as the amnioserosa (Fig. 2D, star). This tissue dramatically changes shape and encompasses most of the dorsal surface of the embryo during a process known as germband retraction. The amnioserosa then constricts, cooperating with a force in the neighboring "leading edge" epithelial cells (Fig. 2D, arrow), which assemble an actin-myosin rich cable around the periphery of the amnioserosa. The combination of amnioserosal and leading edge forces counteracts a resistance force in the more lateral epithelia as the epidermis gradually moves dorsal to cover the amnioserosa in a process known as dorsal closure (Hutson et al 2003; Kiehart et al 2000). In addition to a requirement for actin and myosin, adherens junctions are also needed for dorsal closure (McEwen et al., 2000).

As dorsal closure finishes (Fig. 2E, arrow, time to closure- 15 hours @ 25 degrees C), the embryo begins to resemble the larval body plan. In a process known as head involution, the cells of the head rotate 180 degrees and many of the head structures are internalized (Fig. 2E vs. F). On the ventral epidermis, segmentation is obvious in the form of repeated patterns of actin-rich denticles and naked cuticle (Fig. 2G, arrows). If all goes well, the soon to hatch embryo looks like a first instar larvae (Fig. 2G). By examining the cuticle of this late stage embryo in various mutant backgrounds, specific defects in morphogenesis can be identified. For example, a dorsal hole implicates a gene's role in dorsal closure, while a head hole would point to a function in head involution.

Putting it all together

The stage is set to unravel the complex molecular machinery that rearranges the nuts and bolts of cell and tissue shape to build an animal. For a given candidate regulator, we must understand how this protein interacts with the structural elements and with other regulatory proteins. For a given morphogenetic process, we must understand which regulators are involved and which are dispensable. Studies across several organisms and cell culture models are key to verify observations in one system. As this difficult but rewarding work continues, we will progress from a "parts list" to an "instruction manual" for building an animal.

References

Anastasiadis, P. Z. and Reynolds, A. B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. *J Cell Sci* **113 (Pt 8),** 1319-34.

Bear JE, Svitkina TM, Krause M, Schafer DA, Loureiro JJ, Strasser GA, Maly IV, Chaga OY, Cooper JA, Borisy GG, Gertler FB. (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell* **109**, 509-21.

Beitel, GJ, and Krasnow, MA. (2000). Genetic control of epithelial tube size in the *Drosophila* tracheal system. *Development* **127,** 3271-82.

Bertet, C., Sulak, L. and Lecuit, T. (2004). Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation. *Nature* **429,** 667-71.

Bloor, J. W. and Kiehart, D. P. (2002). Drosophila RhoA regulates the cytoskeleton and cell-cell adhesion in the developing epidermis. *Development* **129,** 3173-83.

Cox, R. T., Kirkpatrick, C. and Peifer, M. (1996). Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during Drosophila embryogenesis. *J Cell Biol* **134,** 133-48.

Craig, R. and Woodhead, J. L. (2006). Structure and function of myosin filaments. *Curr Opin Struct Biol* **16,** 204-12.

Davis MA, Ireton RC, Reynolds AB. (2003). A core function for p120-catenin in cadherin turnover. *J. Cell Biol.* **163.** 525-34.

Drees F, Pokutta S, Yamada S, Nelson WJ, Weis WI. (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* **123.** 903-15.

Dawes-Hoang, R. E., Parmar, K. M., Christiansen, A. E., Phelps, C. B., Brand, A. H. and Wieschaus, E. F. (2005). folded gastrulation, cell shape change and the control of myosin localization. *Development* **132,** 4165-78.

D'Souza-Schorey, C. (2005). Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol* **15,** 19-26.

Gates, J. and Peifer, M. (2005). Can 1000 reviews be wrong? Actin, alpha-Catenin, and adherens junctions. *Cell* **123,** 769-72.

Geisbrecht, E. R., and Montell, D.J. (2002). Myosin VI is required for E-cadherin-mediated border cell migration. *Nat. Cell Biol.* **4.** 616-20.

Grevengoed, E. E., Fox, D. T., Gates, J. and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. *J Cell Biol* **163,** 1267-79.

Grevengoed, E. E., Loureiro, J. J., Jesse, T. L. and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in Drosophila. *J Cell Biol* **155,** 1185-98.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84,** 345-57.

Gumbiner, B. M. (2005). Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol* **6,** 622-34.

Kane DA, McFarland KN, Warga RM. (2005). Mutations in half baked/E-cadherin block cell behaviors that are necessary for teleost epiboly. *Development* **132.** 1105-16.

Kobielak A, Pasolli HA, Fuchs E. (2004). Mammalian formin-1 participates in adherens junctions and polymerization of linear actin cables. *Nat Cell Biol.* **6,** 21-30.

Harrison, S. C. (2003). Variation on an Src-like theme. *Cell* **112,** 737-40.

Hartenstein, V. (1993) in *Atlas of Drosophila Development* (ed. Hatenstein, V.) 2-4 (Cold Spring Harbor Laboratory Press, New York).

Hernandez, S. E., Krishnaswami, M., Miller, A. L. and Koleske, A. J. (2004). How do Abl family kinases regulate cell shape and movement? *Trends Cell Biol* **14,** 36-44.

Hutson MS, Tokutake Y, Chang MS, Bloor JW, Venakides S, Kiehart DP, Edwards GS. (2003). Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. *Science* **5616,** 145-49.

Jacinto, A., Woolner, S. and Martin, P. (2002). Dynamic analysis of dorsal closure in Drosophila: from genetics to cell biology. *Dev Cell* **3,** 9-19.

Kiehart DP, Galbraith CG, Edwards KA, Rickoll WL, Montague RA. (2000). Multiple forces contribute to cell sheet morphogenesis for dorsal closure in Drosophila. *J. Cell Biol.* **149,** 471-90.

Koleske, A. J., Gifford, A. M., Scott, M. L., Nee, M., Bronson, R. T., Miczek, K. A. and Baltimore, D. (1998). Essential roles for the Abl and Arg tyrosine kinases in neurulation. *Neuron* **21,** 1259-72.

Koppen, M., Fernandez, B. G., Carvalho, L., Jacinto, A. and Heisenberg, C. P. (2006). Coordinated cell-shape changes control epithelial movement in zebrafish and Drosophila. *Development* **133,** 2671-81.

Magie, C. R., Pinto-Santini, D. and Parkhurst, S. M. (2002). Rho1 interacts with p120ctn and alpha-catenin, and regulates cadherin-based adherens junction components in Drosophila. *Development* **129,** 3771-82.

McEwen, D. G., Cox, R. T. and Peifer, M. (2000). The canonical Wg and JNK signaling cascades collaborate to promote both dorsal closure and ventral patterning. *Development* **127,** 3607-17.

Moon, S. Y. and Zheng, Y. (2003). Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol* **13,** 13-22.

Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81,** 53-62.

Peifer, M. and Yap, A. S. (2003). Traffic control: p120-catenin acts as a gatekeeper to control the fate of classical cadherins in mammalian cells. *J Cell Biol* **163,** 437-40.

Perez-Moreno, M., Jamora, C. and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* **112**, 535-48.

Planques, V., Warn, A. and Warn, R. M. (1991). The effects of microinjection of rhodamine-phalloidin on mitosis and cytokinesis in early stage Drosophila embryos. *Exp Cell Res* **192,** 557-66.

Pollard, T. D. and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112,** 453-65.

Reinhard, M., Jarchau, T. and Walter, U. (2001). Actin-based motility: stop and go with Ena/VASP proteins. *Trends Biochem Sci* **26,** 243-9.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70,** 401-10.

Ridley AJ, Hall A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70,** 389-99.

Vasioukhin, V., Bauer, C., Yin, M. and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100,** 209-19.

Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP. (2003). Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J Cell Biol.* **163,** 535-45.

Yamada S, Pokutta S, Drees F, Weis WI, Nelson WJ. (2005). Deconstructing the cadherin-catenin-actin complex. *Cell* **123,** 889-901.

Yap, A. S. and Kovacs, E. M. (2003). Direct cadherin-activated cell signaling: a view from the plasma membrane. *J Cell Biol* **160,** 11-6.

Fig. 1.1. Composition of Adherens Junctions. Homophilic, calcium-dependent contact between two cells is mediated by the extracellular domain of Cadherin. Within the cell, Cadherin binds two catenin proteins: at the membrane proximal region, p120-catenin, and more distal, Beta-catenin. Beta-catenin then binds alpha-catenin, which can bind actin. (Adapted from Peifer and Yap, 2003).

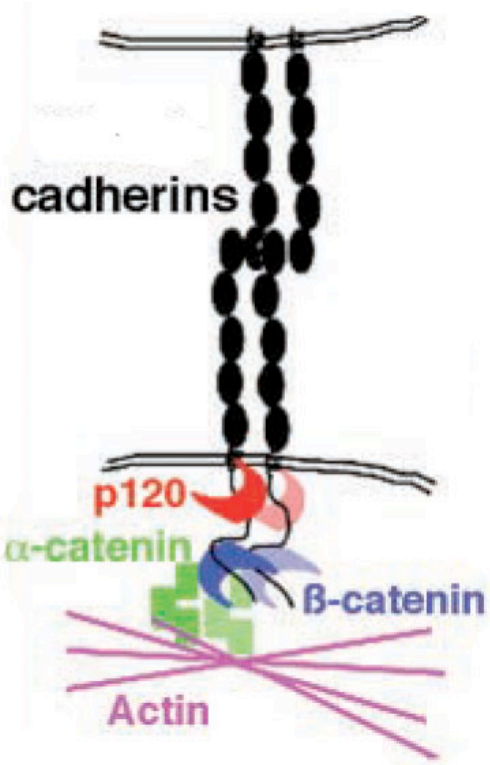
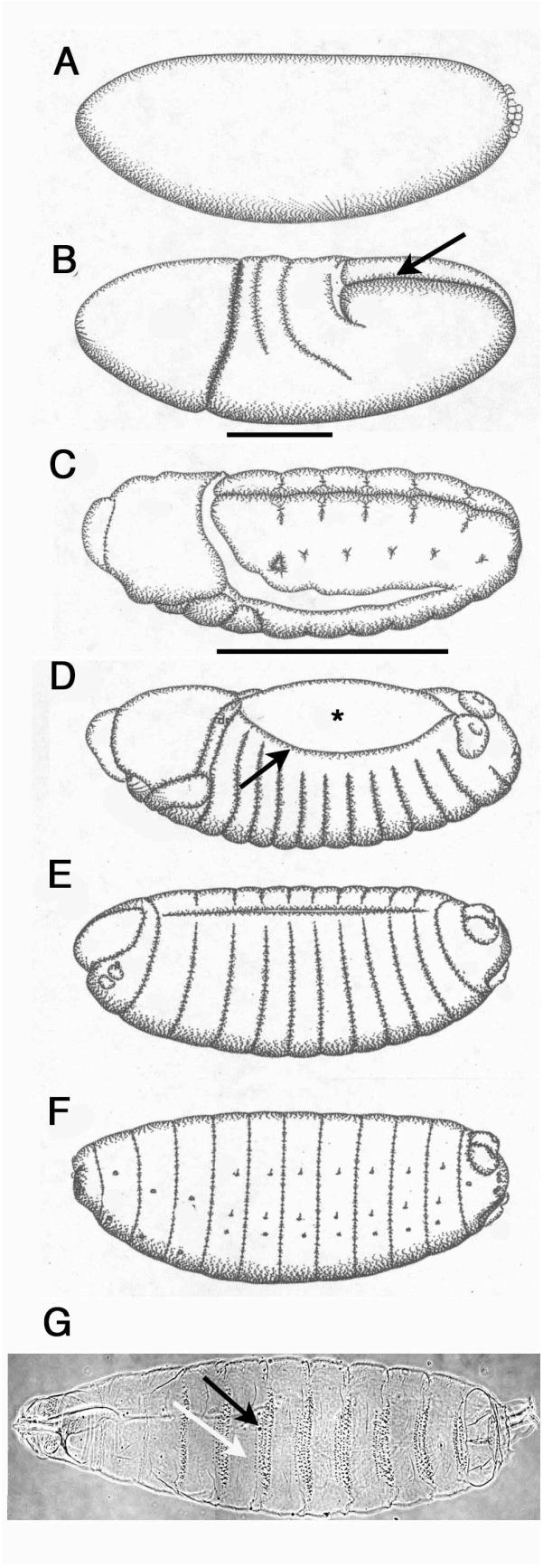

cadherins
p120
α-catenin
ß-catenin
Actin

Fig. 1.2. *Drosophila* epithelial morphogenesis. All embryos are oriented with anterior to the left, dorsal side up (except G, ventral side facing front). A. Blastoderm stage embryo, prior to morphogenesis. B. Gastrulating embryo. Arrow shows site of internalizing posterior midgut. Black line shows approximate length in A-P axis of germband cells. C. Embryo at the completion of germband extension. Black line shows the length of the germband after cell intercalation is complete. Segmentation is apparent, as is the amnioserosa (C-shaped layer of white cells in middle of embryo). D. Embryo after completion of germband retraction. The amnioserosa (star) now covers the dorsal surface of the embryo. Arrow shows location of leading edge cells. E. Embryo after dorsal closure. The amnioserosa is now enclosed. Note the changes in head structure from D., as head involution has begun. F. Embryo at the completion of epithelial morphogenesis. Head involution is now complete (compare with E). G. Cuticle preparation of a wild-type embryo, showing internalized head structures. On the ventral side is the repeated pattern of denticles (black arrow) and naked cuticle (white arrow). A-F adapted from Hatenstein, 1993.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CHAPTER 2

SUMMARY OF CONTRIBUTIONS TO PUBLISHED WORK

Preface

The following describes my contribution to two publications on which I am a co-author.

Myster et al, 2003

This publication addressed the role of the *Drosophila* orthologue of p120-catenin (p120) as an adherens junction component and regulator. Our lab confirmed that like mammalian p120, *Drosophila* p120 binds to the juxta-membrane domain of E-cadherin. Despite reports that mammalian p120 is an essential gene, we found that flies lacking the only p120-like protein are viable and fertile. However, we found that p120 plays a supporting role in adhesion, as reduction of p120 in a background of sensitized adhesion (E-cadherin mutant) enhanced the phenotype.

My contribution to this work concerned the localization of adherens junction components and actin in the absence of p120. I found that the core adherens junction components DE-Cadherin, Armadillo (Beta-catenin) and alpha-catenin localize normally in *p120* mutant embryos. Further, I found no change in the actin cytoskeleton in the absence of p120.

Grevengoed et al, 2003

This publication identified a mechanistic role for Abl kinase in *Drosophila*. To better understand Abl's mechanism of action, we examined *abl* maternal mutants at a simple stage of development- the blastoderm stages. At this stage, no cells have been formed, but nuclei divide and are transiently separated during interphase by "pseudo-cleavage furrows." At the final stage of this process, the nuclei finally form cells in a modified form of cytokinesis known as cellularization. We found *abl* mutants to exhibit defects in both pseudocleavage furrows and at cellularization. Ultimately, we found Abl's regulation of apical actin polymerization to be the cause of this phenotype, and we found that Abl regulates actin by negatively regulating the apical localization of Enabled (Ena).

My role in this work was to analyze the role of adherens junctions during blastoderm stages. As work in our lab had previously found Abl to regulate adherens junctions (Grevengoed et al, 2001), we wondered whether loss of adherens junctions gave rise to an *abl*-like phenotype during blastoderm stages. Thus, I generated maternal mutants for both *armadillo* and *shotgun* (E-cadherin). I found that while either mutant background disrupted adherens junction localization (as assayed by alpha-catenin localization), pseudo-cleavage furrow formation and cellularization occurred normally in the absence of adherens junctions.

References

Grevengoed, E. E., Fox, D. T., Gates, J. and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. *J Cell Biol* **163,** 1267-79.

Grevengoed, E. E., Loureiro, J. J., Jesse, T. L. and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in Drosophila. *J Cell Biol* **155,** 1185-98.

Myster, S. H., Cavallo, R., Anderson, C. T., Fox, D. T., and Peifer, M. (2003). *Drosophila* p120 catenin plays a supporting role in cell adhesion but is not an essential adherens junction component. *J. Cell Biol.* **160**, 433-449.

CHAPTER 3

RHO1 REGULATES *DROSOPHILA* ADHERENS JUNCTIONS INDEPENDENTLY OF P120CTN

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Preface

The following chapter describes work of myself and others regarding the role of Rho1 in adherens junction regulation. The impetus for this work came largely from a publication regarding the role of Rho1 and p120 at adherens junctions (Magie et al 2002). Using RNA interference and mis-expression techniques, this work argued for an essential role for p120 during embryonic development. Further, the authors concluded that p120 is an essential regulator of Rho1, and, in turn, that Rho1 is an essential regulator of adherens junctions.

Given our lab's observation that p120 is not essential for embryonic development or adherens junction function (Myster et al 2003), we decided that further examination was needed to determine if p120 was an essential regulator of Rho1 function. Therefore, we performed the following experiments to ask whether p120 was an essential Rho1 regulator and whether Rho1 itself behaves as a regulator of adherens junctions. In addition to

performing *p120* RNAi and mis-expression approaches, we examined *p120* loss of function. Further, we constructed *Rho1 shg* double mutants to more rigorously evaluate the hypothesis that Rho1 is an adherens junction regulator. While I performed a majority of the experiments, the following were done by other Peifer lab members: C. Homem analyzed the timing of dorsal closure in *p120* mutants and examined *shg* mutant follicle cell clones, S. Myster generated and initiated studies of *p120 Rho1* double mutants, F. Wang assisted in the *p120* RNAi analysis, and E. Bain analyzed cell shape in *p120* mutants and assisted in generating *Rho1 shg* double mutants. This work was published in *Development* in 2005.

Summary

During animal development, adherens junctions (AJs) maintain epithelial cell adhesion and coordinate changes in cell shape by linking the actin cytoskeletons of adjacent cells. Identifying AJ regulators and their mechanisms of action are key to understanding the cellular basis of morphogenesis. Previous studies linked both p120catenin and the small GTPase Rho to AJ regulation and revealed that p120 may negatively regulate Rho. Here we examine the roles of these candidate AJ regulators during *Drosophila* development. We find that although p120 is not essential for development, it contributes to morphogenesis efficiency, clarifying its role as a redundant AJ regulator. Rho has a dynamic localization pattern throughout ovarian and embryonic development. It preferentially accumulates basally or basolaterally in several tissues, but does not preferentially accumulate in AJs. Further, Rho1 localization is not obviously altered by loss of p120 or by reduction of core AJ proteins. Genetic and cell biological tests suggest that p120 is not a major dose-sensitive regulator of Rho1. However, Rho1 itself appears to be a regulator of AJs. Loss of Rho1

results in ectopic accumulation of cytoplasmic DE-Cadherin, but ectopic cadherin does not accumulate with its partner Armadillo. These data suggest Rho1 regulates AJs during morphogenesis, but this regulation is p120-independent.

Introduction

During animal development, coordinated changes in cell shape and position build the body plan and drive morphogenesis. These changes are executed in part by the actin cytoskeleton, and neighboring cells act in concert by linking their cytoskeletons to cell-cell and cell-matrix junctions (reviewed in Perez-Moreno et al., 2003). In epithelial cells, adherens junctions (AJs) mediate cell-cell adhesion, via interactions between cadherins on neighboring cells. Within the cell, the cadherin cytoplasmic tail indirectly interacts with apical actin via β- and α-catenin (reviewed in Tepass et al., 2001).

In addition to these core AJ components, regulatory proteins modulate both AJ stability and connections to the cytoskeleton (reviewed in Gumbiner, 2000). Identifying how these regulators modify AJs during development is critical to understanding morphogenesis. Studies in cultured mammalian cells and other systems identified many candidate AJ regulators, including the catenin p120 and the small GTPase Rho (mammalian RhoA or *Drosophila* Rho1).

p120 binds the Juxta-Membrane region of cadherins (reviewed in Anastasiadis and Reynolds, 2000). Initially, the regulatory relationship between p120 and AJs was unclear. Overexpression of mutant E-cadherins lacking the Juxta-Membrane domain in different mammalian cell types gave opposing results suggesting that p120 either promotes (Yap et al., 1998) or downregulates adhesion (Ozawa and Kemler, 1998). siRNA knockdown of

p120 in mammalian cells clarified this, showing that p120 promotes AJ stability by blocking E-cadherin endocytosis (Davis et al., 2003; Xiao et al., 2003).

In invertebrates, p120 also promotes adhesion but may be dispensable for viability. In *C.elegans, p120/jac-1* RNAi enhances the *hmp-1*/α-catenin phenotype, but *jac-1* RNAi alone does not disrupt morphogenesis (Pettitt et al., 2003). Similarly, loss of p120 enhances the phenotype of *Drosophila* E-Cadherin (DE-Cad) mutants but loss of p120 alone (Myster et al., 2003) or expression of *p120* RNAi transgenes (Pacquelet et al., 2003) do not affect viability or cell adhesion. However, injection of *p120* double-stranded RNA (dsRNA) in embryos was reported to disrupt morphogenesis (Magie et al., 2002). This suggested that rapid depletion of p120 might have more severe consequences than chronic depletion.

In mammalian cells p120 also may function outside of AJs (Anastasiadis and Reynolds, 2000). In the cytoplasm, p120 can negatively regulate RhoA. Rho regulates many cellular processes including actin organization, cell migration, and cell polarity (reviewed in Etienne-Manneville and Hall, 2002). siRNA knockdown of mammalian p120 increases RhoA activity and promotes stress fiber formation (Shibata et al., 2004). Conversely, p120 overexpression causes fibroblasts to lose stress fibers (Anastasiadis et al., 2000; Noren et al., 2000) and contractility (Grosheva et al., 2001), both RhoA-dependent processes (Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996).

Recently, the relationship between p120 and Rho has begun to be tested in vivo, but the results do not yield a consistent mechanistic picture. Embryonic defects caused by knockdown of p120 family members in *Xenopus* can be partially rescued by both dominant-negative (Fang et al., 2004) and wild-type RhoA (Ciesiolka et al., 2004). These contrasting results are consistent with p120 activating or repressing RhoA, respectively. In *Drosophila,*

p120 preferentially binds Rho1-GDP and regulates Rho1 localization. Overexpression of p120 enhances *Rho1*'s phenotype (Magie et al., 2002), suggesting that fly p120 negatively regulates Rho1. Thus p120 may regulate morphogenesis by regulating AJs and/or Rho.

Additionally, Rho itself regulates AJ stability. Blocking RhoA function in keratinocytes prevents the formation of stable AJs (Braga et al., 1997). In *Drosophila*, both *Rho1* loss-of-function and expression of dominant-negative *Rho1* during embryogenesis alter DE-Cad localization (Magie et al., 2002; Bloor and Kiehart, 2002). However, the regulation of AJs by Rho is likely complex, as manipulation of different RhoA effectors can promote or decrease AJ stability in mammalian cells (Sahai and Marshall, 2002, Vaezi et al., 2002). Additionally, AJs may regulate Rho activity, since Cadherin-Cadherin engagement can either activate (Charrasse et al., 2002; Nelson et al., 2004) or inhibit (Noren et al., 2001; 2003) RhoA activity in vitro.

Work in cultured cells supports roles for both p120 and Rho as AJ regulators, but the interactions among p120, Rho, and AJs are complex. Ultimately, we want to understand how Rho and p120 regulate AJs during the intricate events of embryonic morphogenesis. One key question is whether Rho and p120 act together in this process. Here we use *Drosophila* to investigate this.

Materials and methods

RNAi

~0.7 kb of *p120*, *ftz*, or *gfp* coding sequence were amplified from cDNA using primers introducing T7 promoters, and used to synthesize dsRNAs using the MEGAscript® kit (Ambion). For *p120::GFP* injections, the control dsRNA was against *dysfusion* (Jiang and

Crews, 2003). Syncytial blastoderm embryos were bleach-dechorionated, and injected under halocarbon oil at the posterior end with dsRNA at 5µM. For hatch rate estimates, injected *yellow white* embryos were left 3 days at 18°, and unhatched embryos collected for cuticle preparations. Injected *p120::GFP* embryos were aged 24 hours at 18° and transferred to petriPERM plates (Sartorius Corp.) for imaging.

Fly Stocks

Mutations are described at flybase.indiana.edu. $Rho1^{rev220}$ and $Rho1^{1B}$ (Magie et al., 2002; 2005) were from S. Parkhurst (Fred Hutchison). Homozygous *Rho1* mutants were identified using a *Kr::gfp* balancer (Casso et al., 1999); controls were *Kr::gfp* positive siblings. For Rho1 localization in *p120*, *histone::gfp* was the control. Otherwise it was *yellow white*. Recombinant genotypes were confirmed by failure to complement an independently-derived allele; $p120^{308}$ was confirmed by PCR. Cuticle preparations were as in Wieschaus and Nüsslein-Volhard (1986). Unless noted, experiments were done at 25°C. Live imaging utilized wild-type or $p120^{308}$ mutants expressing moesin::GFP. Follicle cell clones: *heat-shock-FLP/+; FRT42D*shg^{R69}*/FRT42D gfp* females were heat-shocked one hour at 37°C for two consecutive days before dissection.

Immunofluorescence

Ovaries were treated as in Magie et al. (2002). Embryos were fixed in 1:1 PBS+3.7% formaldehyde:heptane for 20 min, except for Rho1 staining, which was as in Padash-Barmchi et al. (2005). Embryos were methanol-devitellinized (or hand-devitellenized for phalloidin), blocked and stained in PBS/1% goat serum/0.1%Triton-X-100. Antibodies: anti-Rho1p1D9 (1:50), anti-βPS1 integrin (1:3), anti DE-Cad2 (1:200), anti-ArmN2 (1:200; all Developmental Studies Hybridoma Bank), anti-DRhoGEF2 (1:500, Rogers et al., 2004), anti-

phosphotyrosine (1:1000, Upstate Biotechnology). Alexa®-phalloidin was used at 1:1000; secondary antibodies were Alexas® 488, 568, and 647 (Molecular Probes). Embryos were mounted in Aqua-Polymount (Polysciences). Fixed samples were imaged using a Zeiss LSM510 confocal microscope and LSM software. Live imaging used a Perkin-Elmer Ultraview spinning-disc confocal, an ORCA-ER digital camera (Hamamatsu), and Metamorph software. To analyze dorsal closure timing, we began analysis of all movies when the maximum separation between the leading edges was 52 microns (as measured in Metamorph), and ended analysis when the leading edges met along their entire length. All images were acquired at 40X. Adobe Photoshop7.0® was used to adjust brightness and contrast. When comparing wild-type and mutants, images were adjusted identically.

Results

p120 RNAi is not lethal

Our previous genetic analysis suggested that p120 is not essential for *Drosophila* development, but plays an important positive-modulatory role in cell adhesion that is revealed by reducing levels of DE-Cad (Myster et al., 2003). However, Magie et al. (2002) suggested that embryos suddenly deprived of p120 have developmental defects. We tested this by injecting blastoderm-stage embryos with dsRNA directed against the *p120* coding region. *p120* RNAi does not result in embryonic lethality (68.8% of embryos hatch versus 63.5% of embryos injected with a negative-control (*gfp*) dsRNA; Table 1), while RNAi of the essential gene *ftz* results in embryonic lethality (Table 1). Since about one–third of both *p120*-dsRNA and *gfp*-dsRNA injected embryos died, we suspected this residual lethality resulted from injection-associated defects. Consistent with this, *p120* and *gfp* RNAi gave

similarly variable distributions of cuticle defects (Table 1), while 90% of *ftz* RNAi embryos displayed the characteristic loss of every other body segment (Wakimoto et al., 1984). To rule out the possibility that we failed to knockdown p120 protein, we injected embryos expressing GFP-tagged p120 (Myster et al., 2003), using the same preparation of *p120* dsRNA. >80% of *p120* dsRNA-injected *p120::GFP* embryos lost detectable junctional p120::GFP (Table 2. Fig. 1B; Fig. 9), whereas >80% of *p120::GFP* embryos injected with control dsRNA retained junctional p120::GFP (Table 2; Fig. 1A; Fig. 9). Thus RNAi of *Drosophila p120*, like *p120* loss-of-function mutations, is not lethal.

Loss of p120 slows but does not disrupt dorsal closure

p120 mutants are viable and fertile, and AJ proteins and actin accumulate normally in *p120* mutant embryos (Myster et al., 2003). We noted, in passing, that a fraction of *p120* mutants exhibit slight defects in cell shape during dorsal closure, a process during which lateral sheets of epithelial cells elongate and migrate dorsally, enclosing the embryo in epidermis. To examine whether p120 plays a subtle role in morphogenesis, we looked in detail at dorsal closure, examining cell shape changes and cell behavior in fixed and live *p120* mutants. We examined each stage in dorsal closure and observed no consistent differences between *p120* mutants and wild-type (Fig. 1C-J). F-actin localization was also similar (Fig. 1 K vs. L).

We next examined whether more subtle changes on cell behavior were revealed by imaging dorsal closure in living embryos expressing a GFP-tagged F-actin-binding fragment of Moesin that highlights the cytoskeleton (Moesin::GFP; Edwards et al., 1997). Once again, we saw no gross defects in cell shape in *p120* mutants. However, loss of p120 slows the rate of dorsal closure. On average, wild-type embryos complete dorsal closure within 75

minutes (Fig. 1O). *p120* mutants are significantly slower, taking 112 minutes (Fig. 1O). Despite this, *p120* mutants complete dorsal closure without apparent defects. Thus, loss of p120, while not lethal, alters the efficiency of morphogenesis.

Rho1 exhibits dynamic localization

In mammalian cells, p120 regulates Rho activity. One mechanism to regulate Rho is by controlling its subcellular localization. Previous workers examined Rho1 localization in both ovaries and embryos (Magie et al., 2002; Padash-Barmchi et al., 2005). *Drosophila* Rho1 was reported to localize to AJs (Magie et al., 2002), suggesting that this might be a mechanism by which it both regulates and is regulated by AJs. We re-examined Rho1 localization compared to that of AJs. This revealed new information about Rho1dynamic localization, sometimes confirming previous work and in other cases contradicting it.

We began with oogenesis (utilizing the same protocol and anti-Rho1 monoclonal antibody used in Magie et al, 2002). Ovarian follicle cells form an epithelium with its apical surface inward, providing an excellent place to examine AJs. After egg chambers form, Rho1 localizes to follicle cell apical and lateral membranes (Fig. 2A, white arrowhead), and along lateral membranes of stalk cells (Fig. 2A, red arrowhead). Rho1 remains enriched at follicle cell lateral membranes (Fig. 2F,H), but apical enrichment decreases at later stages (Fig. 2H). DE-Cad is strongly enriched in apical AJs (Fig. 2C,F,H blue arrowheads). Rho1 localization sometimes overlaps DE-Cad at AJs, but it is not enriched there (Fig. 2C,F,H). In grazing sections at stage 10, Rho1 accumulates in puncta where three follicle cells meet (Fig. 2E arrows, J); these puncta are basal to the strongest DE-Cad staining (data not shown) and do not co-localize with DE-Cad (Fig. 2E). Thus Rho1 is not enriched in AJs of most follicle

cells. However, like AJ proteins (Peifer et al., 1993; Oda et al., 1997), Rho1 does accumulate at germ cell boundaries (Magie et al., 2002; Fig. 2B arrowhead).

Posterior polar follicle cells preferentially accumulate AJ proteins (Peifer et al., 1993; Niewiadomska et al., 1999; Fig. 2C, arrow). In contrast, Rho1 levels in these cells resemble those of their neighbors (Fig. 2C, arrow). Border cells also accumulate elevated levels of AJ proteins (Oda et al., 1997; Niewiadomska et al., 1999) and require DE-Cad to migrate to the anterior end of the oocyte. During migration, border cells accumulate Rho1 (Magie et al., 2002; Fig. 2L) at both the plasma membrane and in cytoplasmic puncta (Fig. 2M arrowheads). These may represent multivesicular bodies that are enriched in border cells (Peifer et al., 1993). DE-Cad is enriched in apical AJs of centripetal follicle cells that migrate between nurse cells and the oocyte (Fig. 2D; red arrowhead). Rho1 accumulates with DE-Cad in these cells apically (Fig. 2D; red arrowhead); Rho1 is also enriched in more basal puncta (Fig. 2D; blue arrowhead). Thus in the ovary, while Rho1 and DE-Cad overlap in some places, Rho1 does not preferentially localize to AJs.

We also compared Rho1 and DE-Cad localization in embryos, extending earlier work (Magie et al., 2002; Padash-Barmchi et al., 2005; the pictures presented use the protocol of Padashi-Barchi (2005), but similar results were also seen with the protocol of Magie et al. (2002)). Rho1 localization is very dynamic. During cellularization, Rho1 is enriched at invaginating furrow canals (Fig. 3A, blue arrowhead), as previously observed (Padash-Barmchi et al., 2005), while DE-Cad localizes both to basal (Fig. 3A, yellow arrowhead) and developing apical AJs (Fig. 3A, white arrowhead). At gastrulation, DE-Cad is enriched in AJs of posterior midgut cells (Fig. 3E, blue arrowhead), while Rho1 accumulates in basal puncta (Fig. 3D,E white arrowheads) that may be furrow canal remnants. DE-Cad is also

enriched in apical AJs of invaginating cells in the ventral furrow, while Rho1 is only weakly enriched in the ventral furrow (Fig. 3F, arrowheads). After mesodermal cells invaginate, they accumulate cortical Rho1 (Fig. 3H, yellow arrowheads). In epithelial cells, we observed two general features of Rho1 localization. In several cell types, Rho1 localized basally. After germband extension, Rho1 accumulates basally where ectoderm meets mesoderm (Fig. 3G,H arrowheads). ßPS1-integrin also localizes there (Fig. 3I, arrowheads). Later, Rho1 localizes basally in the hindgut epithelium (Fig. 3M, blue arrowheads; DE-Cad accumulates apically). Second, comparison of blastoderm, extended-germband, and dorsal closure-stage embryos shows that cortical enrichment of Rho1 decreases over time (Fig. 3J-L). Thus Rho1 localization varies in different cell types, but it is not significantly enriched in AJs of most cells.

Rho1 zygotic mutants have defects during dorsal closure (Magie et al., 1999). We thus closely examined Rho1 localization at that stage, collecting sections in the Z-axis through the lateral epithelia. Apically, Rho1 does not localize to AJs but accumulates at low levels in the cytoplasm (Fig. 4A,A'). Basal to AJs (where DE-Cad is seen at AJs of invaginating segmental groove cells; Fig. 4B,B'), Rho1 levels increase and it is more cortical. Thus in these cells Rho1 is enriched basal to AJs. Another important input in Rho1 regulation is localized activation by RhoGEFs. While a comprehensive study is beyond the scope of our work, we examined RhoGEF2 during dorsal closure, which colocalizes with Rho1 during cellularization (Padash Barmchi et al., 2005). During dorsal closure, RhoGEF2 accumulates basal to AJs (Fig. 4H-H'), like Rho1. However, RhoGEF2 is more cortical, poising it to activate a cortical pool of Rho1. Thus, during oogenesis and embryogenesis Rho1 localization varies among different cell types, with basal or basolateral accumulation in

many epithelia. Importantly, we found no evidence for preferential Rho1 accumulation at AJs, although the localizations do sometimes overlap.

Rho1 localization is independent of p120 and core AJ protein function.

Both p120 and α-catenin can bind Rho1 (Magie et al., 2002). These authors reported that RNAi of either *p120* or *α-catenin* altered Rho1 localization. While we did not observe strong enrichment of Rho1 at AJs, it remained possible that p120 or core AJ proteins could regulate Rho1 localization. To examine this, we first examined *p120* null mutant ovaries and embryos. As previously observed in embryos (Myster et al., 2003), *p120* mutant follicle cells exhibit normal DE-Cad localization to AJs (Fig. 2G,I). In *p120* mutant follicle cells, Rho1 localization to the cytoplasm, the lateral membrane (Fig. 2F vs. G, H vs. I) and its accumulation in puncta in late-stage follicle cells (Fig. 2J vs. K) were indistinguishable from wild-type. We also saw no change in Rho1 localization in *p120* mutant embryos; e.g., Rho1 localization to furrow canals (Fig. 3B vs. C) and its basal localization in the hindgut (Fig. 3M vs. N) are unchanged. Further, there were no changes in apical (Fig. 4C' vs. A') or basolateral (data not shown) Rho1 localization during dorsal closure in *p120* mutants. In particular, we did not observe elevated Rho1 accumulation at the leading edge, as was previously reported for *p120* RNAi (Magie et al., 2002). Thus p120 is not required for normal Rho1 localization in ovarian or embryonic epithelia.

We next examined whether core AJ proteins are critical for Rho1 localization. We addressed this in the follicle cell epithelium by generating homozygous null DE-Cad (*shotgun;shg*^{R69}) mutant clones (marked by loss of GFP; Fig. 4I-M''). DN-Cadherin can act in place of DE-Cad (Tanentzapf et al., 2000) during early oogenesis; thus in early stage egg chambers Arm remains at AJs despite loss of DE-Cad (Fig. 4I). However, by stage 10 this

redundancy is less apparent; loss of DE-Cad substantially reduces Arm accumulation in AJs (Fig. 4J-K''). We thus confined our analysis to late stages. Rho1 localization is unchanged in *shg* null mutant follicle cells (Fig. 4L,M). We also examined Rho1 localization in embryos zygotically mutant for core AJ proteins. In zygotic *arm* null mutants, DE-Cad is no longer detectable by dorsal closure (data not shown). Zygotic *shg* null mutants lose DE-Cad from the epidermis, though some maternal DE-Cad remains in the aminoserosa (Fig. 4G'). In both *arm* (Fig. 4E vs. F) and *shg* (Fig. 4G) zygotic mutants, Rho1 localization is unchanged. Thus core AJ components do not play a major role in Rho1 localization in *Drosophila* epithelia.

***Drosophila* p120 is not a key regulator of Rho1 function.**

While p120 is not essential for Rho1 localization, it might regulate Rho1 function by other mechanisms. Often regulators, even those that are partially-redundant, can be identified by looking for phenotypic effects in a sensitized genetic background. For example, p120's supporting role in AJs was revealed by the fact that loss of p120 strongly enhances DE-Cad mutants (Myster et al., 2003). In zygotic *Rho1* mutants, maternal Rho1 is gradually depleted, and thus we reasoned that altering a Rho1 regulator might modify the consequences of reduced levels of Rho1.

To test whether *p120* and *Rho1* genetically interact, we generated *p120 Rho1* double mutants and compared their phenotypes to those of *Rho1*. *Rho1* mutant embryos had the defects described previously (Magie et al., 1999; unless noted, we used the strong allele $Rho1^{rev220}$). Most embryos lack head cuticle; the remaining cuticle differs significantly in size among different embryos (Fig. 5 and Table 3), perhaps due to the varying degrees of

"bowing" of the dorsal surface previously observed (Magie et al., 1999). *p120* null mutants (*p120*^{308}, used for all experiments) have normal cuticles and are embryonic viable.

Complete removal of *p120* does not substantially modify the *Rho1* phenotype, nor was a novel double-mutant phenotype seen. The percentage of embryos with head holes was quite similar, for example (76.1% of *Rho1* mutants versus 74.6% of *p120 Rho1* double mutants; Table 3). We did observe an effect on one aspect of the phenotype: *p120 Rho1* double-mutants had twice as many long cuticles than *Rho1* single mutants (Table 3; Fig. 5; this was seen in two separate experiments). This phenotypic shift may reflect subtle suppression of *Rho1* by *p120*. However, this is much more subtle than the effect of p120 loss on the *shg* phenotype (Myster et al., 2003), and may simply reflect differences in genetic background or sample preparation—we observed comparable variation between different cuticle preparations of the same genotype (data not shown).

As a second test for genetic interactions, we examined whether p120 over-expression modifies the *Rho1* phenotype, ubiquitously expressing a p120 transgene under the control of the GAL4-UAS system (Myster et al., 2003; using actin-GAL4). Magie et al. (2002) previously reported that *p120* over-expression using actin-GAL4 enhanced the *Rho1* phenotype. However, using an independently-derived UAS-p120 transgene, we did not observe phenotypic enhancement. Instead, our results suggest a slight suppression of the *Rho1* phenotype. The overall range of phenotypes was similar, with a small shift toward the less severe categories (Table 3; data not shown). However, this effect was fairly small, and may reflect differences in genetic background.

We also compared the effect of loss of Rho1 with the loss of both Rho1 and p120 on F-actin during dorsal closure. As previously observed by Magie et al. (1999), *Rho1* mutants

are nearly normal during early dorsal closure (Fig. 5J vs. K), with defects becoming apparent during late dorsal closure. The *Rho1* phenotype is variable-- in more severely affected *Rho1* mutants both the leading-edge actin cable and cell shape changes are less uniform (Fig. 5M vs. N). In less severely affected mutants, when leading edges meet at the dorsal midline the two epithelial sheets do not line up or intercalate normally (Magie et al., 1999; Fig. 5P vs. Q). *p120 Rho1* double mutants exhibited the same range of phenotypes as *Rho1* single mutants during early (Fig. 5K vs. L) and later stages of dorsal closure (Fig. 5N vs. O, Q vs. R). Thus, *p120* does not behave genetically as a key regulator of Rho1 function, contrasting with its strong genetic interactions with DE-Cad (Myster et al., 2003).

***Rho1* and *shg* genetically interact.**

These data suggest that p120 is not a required regulator of Rho function in *Drosophila*. However, there are other strong links between Rho and AJs-- evidence from mammalian cell culture and *Drosophila* strongly suggest that Rho regulates AJ protein localization and function. To probe the relationship between Rho and AJs during embryonic morphogenesis, we asked whether *Rho1* genetically interacts with DE-Cad (*shg*) by constructing double mutants for *Rho1* and each of three *shg* alleles: a weak allele, shg^{G119} (Tepass et al., 1996), a strong allele, shg^{2} (Uemura et al., 1996=shg^{IH81}, Nüsslein-Volhard et al. 1984), and a protein-null allele, shg^{R69} (Godt and Tepass, 1998). Embryonic epithelia have differential sensitivity to DE-Cad loss (Tepass et al., 1996). The head epidermis, which undergoes extensive rearrangements, is most sensitive. Thus, weak alleles like shg^{G119} mainly disrupt head cuticle (Fig. 6, Table 4). The next most sensitive tissue is the ventral epidermis, site of neuroblast delamination. Strong *shg* alleles like shg^{2} disrupt head and, to varying degrees, ventral epidermis (Fig. 7, Table 5). Finally, null *shg* alleles like shg^{R69} lack

head, ventral, and to some extent, dorsal cuticle (Fig. 7, Table 5). We reasoned that novel phenotypes in double mutants or suppression of one mutation by another might indicate a genetic interaction.

Loss of *Rho1* enhances the phenotype of the weak allele shg^{G119} (Fig. 6, Table 4). Most double mutant embryos have holes in their ventral epidermis that are not present in most shg^{G119} single mutants. Since both *Rho1* and shg^{G119} affect head involution, the enhancement of this phenotype may simply reflect additive effects. However, since loss of Rho1 does not affect integrity of the ventral epidermis, we believe enhancement of this aspect of the phenotype is likely to reflect a genetic interaction. We also assessed whether reduction of the dose of Rho1 had an effect on the shg^{G119} phenotype—there was no apparent effect of reducing the maternal and zygotic Rho1 dose by half. Surprisingly, however, *Rho1* does not enhance, but instead partially suppresses, the phenotype of stronger *shg* alleles (Fig. 7, Table 5). For both alleles, a larger fraction of double mutant progeny fall into less severe phenotypic categories. Thus, *Rho1* behaves genetically as a regulator of AJ function, but the nature of this genetic interaction is complex.

We also sequenced the two non-null *shg* alleles. shg^{G119} has an in-frame deletion of four conserved amino acids in the membrane-proximal lamininG domain in the extracellular domain (Fig 6D). shg^{2} has two mutations: missense changes in a conserved amino acid in the lamininG domain and in a conserved residue in the cytoplasmic tail (Fig. 6D, 7G) at the C-terminal end of the Arm-binding site (Pai et al. 1996; Huber and Weis, 2001; Pokutta and Weis, 2000).

Rho1 regulates DE-Cad but not Arm localization

Rho1 was reported to be required for correct localization of AJ proteins (Magie et al., 2002; Bloor and Kiehart, 2002). We examined embryos lacking zygotic Rho1. During dorsal closure, DE-Cad accumulated ectopically, as previously reported (Magie et al., 2002). Ectopic DE-Cad accumulates in the cytoplasm of epithelial or amnioserosal cells near the leading edge (Fig. 8A vs. B). Ectopic DE-Cad also accumulates in *Rho1* embryos prior to (Fig. 8C vs. D) and following dorsal closure (Fig. 8E vs. F). Importantly, however, ectopic DE-Cad did not colocalize with its binding partner Arm (Fig. 8B). Finally, we used the ectopic DE-Cad phenotype of *Rho1* mutants to further test whether p120 regulates Rho1. *p120 Rho1* double mutants accumulate ectopic DE-Cad during dorsal closure in a fashion identical to *Rho1* single mutants (Fig. 8G vs. B). Thus, Rho1 regulates DE-Cad but not Arm localization and the effect of loss of Rho1 is not enhanced or suppressed by removing p120.

Discussion

The regulation of adhesion and its coupling to the actin cytoskeleton are critical for morphogenesis. Rho and p120 both regulate adhesion and the cytoskeleton, but the precise nature of their roles and the interrelationship among them are less clear. We addressed these issues during development in *Drosophila*.

Drosophila p120: a redundant regulator of AJs

In mammalian cells, p120 is a key regulator of cadherin-based adhesion (Davis et al., 2003; Xiao et al., 2003). However, the universality of this role was called into question by the viability of *p120* mutant flies (Myster et al., 2003). One caveat remained, however. Magie et al. (2002) reported that rapidly depleting fly embryos of p120 by RNAi led to defects in morphogenesis and Rho1 localization. To resolve this discrepancy, we carried out

p120 RNAi. Our data confirm that knockdown of p120 does not result in lethality. Thus, *Drosophila* (these data; Myster et al., 2003; Pacquelet et al., 2003) and *C. elegans* p120 (Pettitt et al., 2003) are dispensable for development. In contrast, p120 knockdown in *Xenopus* or *M. musculus* is lethal (Fang et al., 2004; Al Reynolds, pers. comm.), suggesting differences in p120's importance in vertebrates versus invertebrates.

Since *Drosophila* has a single p120 family member, simple redundancy does not explain the difference. We imagine two possible explanations. First, p120 proteins may play fundamentally different roles in vertebrates and invertebrates. Alternately, p120's role in both may be similar, but the relative importance of p120 and unrelated, partially-redundant regulators of cadherin and/or Rho may differ. We favor the latter possibility, since p120 binds to and promotes the function of AJs in vertebrates and invertebrates (Anastasiadis and Reynolds, 2000; Myster et al., 2003; Pettitt et al., 2003), and p120 has a conserved role in regulating morphogenesis, contributing to dorsal closure efficiency (these data) and regulating dendrite morphology (Li et al. 2005) in *Drosophila* and regulating gastrulation and craniofacial morphogenesis in *Xenopus* (Fang et al., 2004; Ciesiolka et al., 2004). One role of p120 is to inhibit cadherin endocytosis. Perhaps in invertebrates other regulators of cadherin trafficking compensate for its absence.

Drosophila p120: a redundant regulator of Rho1?

The second postulated role for p120 is as a Rho regulator. The viability of *p120* mutants suggested that *Drosophila* p120 is not an essential Rho1 regulator. However, this did not rule out a role as one of several Rho1 regulators with overlapping functions. For example, Magie et al. (2002) suggested overlapping roles for p120 and α-catenin, with p120 regulating Rho1 localization during dorsal closure. We thus looked for dose-sensitive genetic

interactions between *p120* and *Rho1*. Loss of p120 does not substantially affect Rho1 function, as assessed by cuticle phenotype. Further, loss of p120 does not enhance or suppress the effect of *Rho1* on F-actin or DE-Cad localization during dorsal closure. p120 over-expression had only a slight effect on the *Rho1* phenotype, a result that may reflect variation in genetic background. Thus, although *Drosophila* p120 preferentially binds inactive Rho1 (Magie et al., 2002), it is not a major dose-sensitive regulator of Rho1.

We also tested the hypothesis that p120 regulates Rho1 localization, examining several places where Rho1 exhibits striking subcellular localization, and examining the place where Rho1 exhibits its zygotic phenotype—the dorsal closure front. We saw no change in Rho localization in *p120* mutants. Therefore, if p120 regulates Rho1 localization, it must do so redundantly with other putative Rho1 regulators, such as α-catenin (Magie et al., 2002). These data do not rule out the possibility that p120 recruits a pool of active Rho1, which may be only a small fraction of total cellular Rho1.

p120 appears to regulate RhoA during *Xenopus* development (Fang et al., 2004; Ciesiolka et al., 2004). Perhaps redundant Rho regulators act in parallel to p120 in flies. Alternatively, p120's role as a Rho regulator may not be conserved—p120's N-terminal domain, which is implicated in regulating transitions between its adhesive and cytoplasmic roles, is not well conserved between mammalian and fly p120. Since *p120* (Myster et al., 2003) and *Rho1* (Fig. 5) mutations modify *shg* mutant phenotypes differently, p120 and Rho1 may act in separate pathways to regulate AJs in *Drosophila*.

Rho1 localization and its regulation

We extended previous analyses of Rho1 localization (Magie et al., 2002; Padash-Barmchi et al., 2005). It is dynamic, with Rho1 accumulating at different subcellular sites in

distinct cell types, some consistent with proposed Rho functions. For example, mammalian RhoA regulates integrin-based cell-matrix junctions (reviewed in Burridge and Wennerberg, 2004.). Rho1's basal localization raises the possibility that it may regulate integrins in *Drosophila*. Rho1 accumulation in mesodermal cells is consistent with its role in regulating cell shape during mesoderm spreading (Wilson et al., 2005). Relative levels of cortical Rho1 decrease through development. Perhaps at later stages Rho1 is activated by localized RhoGEFs. Consistent with this, RhoGEF2 is more cortically enriched during dorsal closure than Rho1. Thus, future studies will need to examine the localization of Rho1 regulators and effectors. Recent advances also allow visualization of active Rho GTPases (e.g., Benink and Bement, 2005). As much of the Rho1 pool may be inactive, application of this approach to flies will advance our understanding of Rho1 function.

It was previously proposed that Rho1 is enriched at *Drosophila* AJs and that this is regulated by core AJ proteins (Magie et al., 2002). We examined this in follicle cells and embryos. In follicle cells, Rho1 localizes to lateral and apical membranes in early egg chambers, and to lateral membranes later. In neither case did we observe enrichment in AJs, although Rho1 is not excluded from them. In embryonic epithelia, Rho1 sometimes localized uniformly to the basolateral membrane while in other places it was enriched basally. During dorsal closure, when *Rho1* exhibits its zygotic phenotype, Rho1 accumulates basal to AJs. The lack of preferential Rho1 localization at AJs does not rule out accumulation of a pool of active Rho1 at AJs- this will require reagents to measure Rho1 activation in vivo. We also tested the hypothesis that AJs regulate Rho1 localization. In follicle cells mutant for DE-Cad and embryos mutant for *arm* or DE-Cad during dorsal closure, Rho1 localization was not obviously disturbed.

Rho is an important regulator of AJs during embryonic morphogenesis

In cultured cells, Rho and AJs have a complex relationship. Rho regulates AJ stability and conversely AJs regulate Rho activity (reviewed in Yap and Kovacs, 2003). Further, different Rho effectors can promote or decrease AJ stability in cultured mammalian cells (Sahai and Marshall, 2002, Vaezi et al., 2002). We examined this complex relationship during morphogenesis, using genetic and cell biological assays. Our data support the hypothesis that Rho1 is an important regulator of cadherin-based adhesion during embryonic development.

Loss of Rho1 leads to DE-Cad mislocalization (Magie et al., 2002), while dominant-negative Rho1 reduces DE-Cad in AJs (Bloor and Kiehart, 2002), implicating Rho1 in regulating DE-Cad localization. Our results support this hypothesis. Cytoplasmic DE-Cad accumulation is consistent with a role for Rho1 in regulating either DE-Cad transport to or recycling from AJs. We observed that the ectopic DE-Cad in *Rho1* mutants accumulates independently of its binding partner Arm. In mammalian cells, newly-synthesized E-cadherin must bind β-Catenin before it can be transported to AJs (Chen et al., 1999), while endocytosed E-cadherin accumulates with either no (Xiao et al., 2003) or reduced (Le et al., 1999) amounts of β-Catenin. Thus our data are more consistent with ectopic DE-Cad accumulating after endocytosis. Consistent with this, mammalian RhoA regulates clathrin-mediated endocytosis (Lamaze et al., 1996), and *Drosophila* Rho1 regulates endocytosis of the ligand Wingless (Magie et al., 2005). Further, constitutively-active Rac1, which can inhibit RhoA (Sander et al., 1999), triggers E-cadherin recruitment to intracellular vesicles in keratinocytes (Akhtar and Hotchin, 2001). Since high levels of Rho1 do not accumulate at AJs, either a small pool of active Rho1 at AJs is sufficient to inhibit cadherin endocytosis or the effect is more indirect, with Rho1 acting on the actin cytoskeleton or regulators of

endocytic trafficking. The mechanism by which Rho1 regulates DE-Cad trafficking is an interesting question for future studies.

Mammalian p120 also regulates cadherin endocytosis (Davis et al., 2003; Xiao et al., 2003). The viability of fly *p120* mutants suggests that in flies this role is not rate limiting, though the enhancement of mutants with reduced DE-Cad by *p120* is consistent with p120 playing a similar role (Myster et al. 2003). p120 and Rho could regulate DE-cad trafficking via the same or distinct pathways. The effect on DE-Cad trafficking in zygotic *Rho1* mutants, which should have limiting levels of maternal Rho1, is not enhanced by removing *p120*. This is more consistent with a model in which the two proteins work in different pathways, and in which p120 acts partially redundantly with another unknown regulator.

Our analysis of *Rho1* and *Rho1 shg* mutants is consistent with the hypothesis that Rho1 regulates AJs, but suggests that their interactions are complex. A weak *shg* allele was enhanced but stronger alleles were suppressed. There are several possible explanations for these contrasting results. Weak alleles (e.g. shg^{G119}) make protein with reduced but residual function. If Rho1 negatively-regulates cadherin endocytosis, more mutant DE-Cad protein might be endocytosed in Rho1's absence, further reducing functional DE-Cad and enhancing the phenotype. However, null or very strong *shg* alleles accumulate no functional DE-Cad at AJs (for shg^{2} see Uemura et al. 1996), rendering regulation of cadherin endocytosis moot. The slight suppression by *Rho1* of strong *shg* alleles may result from a reduction of morphogenetic movements, reducing cuticle disruption (as in Tepass et al., 1996). Alternately, some mutant DE-Cad proteins may be capable of coupling to Rho1 while others are not. Rho1 can bind α-catenin (Magie et al., 2002), and active Rho1 may be recruited to AJs by that interaction. shg^{G119} has a wild-type cytoplasmic domain and could presumably

couple to Rho1; reducing Rho1 might further impair its function. In contrast, the *shg*^{2} mutation may impair Arm and/or α-catenin binding and thus Rho1 recruitment; if so this mutant protein wouldn't be further impaired by Rho1 removal. Finally, the complex genetic interactions might reflect different requirements for Rho1 during neuroblast delamination and head involution, which are affected by strong or weak reduction in DE-Cad function, respectively (Tepass et al., 1996). Future studies of Rho regulation of and by AJs will help distinguish between these possibilities.

References

Akhtar, N., and Hotchin, N. A. (2001). RAC1 regulates adherens junctions through endocytosis of E-cadherin. *Mol. Biol. Cell* **12**, 847-862.

Anastasiadis, P. Z., Moon, S. Y., Thoreson, M. A., Mariner, D. J., Crawford, H. C., Zheng, Y., and Reynolds, A. B. (2000). Inhibition of RhoA by p120 catenin. *Nat. Cell Biol.* **2**, 637-644.

Anastasiadis, P. Z., and Reynolds, A. B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. *J. Cell Sci.* **113**, 1319-1334.

Benink, H. A., and Bement, W. M. (2005). Concentric zones of active RhoA and Cdc 42 around single cell wounds. *J. Cell Biol.* **168,** 429-439.

Bloor, J. W., and Kiehart, D. P. (2002). *Drosophila* RhoA regulates the cytoskeleton and cell-cell adhesion in the developing epidermis. *Development* **129**, 3173-3183.

Braga, V. M., Machesky, L. M., Hall, A., and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* **137**, 1421-1431.

Burridge, K., and Wennerberg, K. (2004). Rho and Rac take center stage. *Cell* **116**, 167-179.

Casso, D., Ramirez-Weber, F., and Kornberg, T. B. (1999). GFP-tagged balancer chromosomes for *Drosophila melanogaster*. *Mech. Dev.* **88**, 229-232.

Charrasse, S., Merian, M., Comunale, F., Blangy, A., and Gauthier-Rouviere C. (2002). N-cadherin-dependent cell-cell contact regulates Rho GTPases and beta-catenin localization in mouse C2C12 myoblasts. *J. Cell Biol.* **158**, 953-965.

Chen, Y. T., Stewart, D. B., and Nelson, W. J. (1999). Coupling assembly of the E-cadherin/beta-catenin complex to efficient endoplasmic reticulum exit and basal-lateral membrane targeting of E-cadherin in polarized MDCK cells. *J. Cell Biol.* **144**, 687-699.

Ciesiolka, M., Delvaeye, M., Van Imschoot, G., Verschuere, V., McCrea, P., van Roy, F., and Vleminckx, K. (2004). p120 catenin is required for morphogenetic movements involved in the formation of the eyes and the craniofacial skeleton in *Xenopus*. *J. Cell Sci.* **117**, 4325-4339.

Chrzanowska-Wodnicka, M., and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* **133,** 1403-1415.

Davis, M. A., Ireton R. C., and Reynolds, A. B. (2003). A core function for p120-catenin in cadherin turnover. *J. Cell Biol.* **163**, 525-534.

Edwards, K.A., Demsky, M., Montague, R.A., Weymouth, N., Kiehart, D.P. (1997). GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in *Drosophila*. *Dev Biol*. **191**,103-17.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.

Fang, X., Ji, H., Wan Kim, S. W., Park, J. I., Vaught, T. G., Anastasiadis, P. Z., Ciesiolka, M., and McCrea, P. D. (2004). Vertebrate development requires ARVCF and p120 catenins and their interplay with RhoA and Rac. *J. Cell Biol.* 165, 87-98.

Godt, D., and Tepass, U. (1998). *Drosophila* oocyte localization is mediated by differential cadherin-based adhesion. *Nature* **395**, 387-391.

Grosheva, I., Shtutman, M., Elbaum, M., and Bershadsky, A. D. (2001). p120 catenin affects cell motility via modulation of activity of Rho-family GTPases: a link between cell-cell contact formation and regulation of cell locomotion. *J. Cell Sci.* **114**, 695-707.

Gumbiner, B. M. (2000). Regulation of cadherin adhesive activity. *J.Cell Biol.* **148**, 399-404.

Huber, A.H., and Weis, W.I. (2001). The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. *Cell* **105**, 391-402.

Jiang, L., and Crews, S. T. The *Drosophila dysfusion* basic helix-loop-helix (bHLH)-PAS gene controls tracheal fusion and levels of the trachealess bHLH-PAS protein. (2003). *Mol. Cell Biol.* **23**, 5625-5637.

Lamaze, C., Chuang, T. H., Terlecky, L. J., Bokoch, G. M., and Schmid, S. L. (1996). Regulation of receptor-mediated endocytosis by Rho and Rac. *Nature* **382**, 177-179.

Le, T. L., Yap, A. S., and Stow, J. L. (1999). Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. *J. Cell Biol.* **146**, 219-232.

Li, W., Li, Y., and Gao, F.-B. (2005). Abelson, enabled and p120catenin exert distinct effects on dendritic morphology in Drosophila. *Dev. Dyn.* Jul 7 Epub ahead of print.

Magie, C. R., Meyer, M. R., Gorsuch, M. S., and Parkhurst, S. M. (1999). Mutations in the Rho1 small GTPase disrupt morphogenesis and segmentation during early *Drosophila* development. *Development* **126**, 5353-5364.

Magie, C. R., Pinto-Santini, D., and Parkhurst, S. M. (2002). Rho1 interacts with p120ctn and α-catenin, and regulates cadherin-based adherens junction components in *Drosophila*. *Development* **129**, 3771-3782.

Magie, C. R., and Parkhurst, S. M. (2005). Rho1 regulates signaling events required for proper *Drosophila* embryonic development. *Dev. Biol.* **278**, 144-154.

Myster, S. H., Cavallo, R., Anderson, C. T., Fox, D. T., and Peifer, M. (2003). *Drosophila* p120 catenin plays a supporting role in cell adhesion but is not an essential adherens junction component. *J. Cell Biol.* **160**, 433-449.

Nelson, C. M., Pirone, D. M., Tan, J. L., and Chen, C. S. (2004). Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. Mol. Biol. Cell 15, 2943-2953.

Niewiadomska, P., Godt, D., and Tepass, U. (1999). DE-Cadherin is required for intercellular motility during *Drosophila* oogenesis. *J. Cell Biol.* **144**, 533-547.

Noren, N. K., Liu, B. P., Burridge, K., and Kreft, B. (2000). p120 catenin regulates the actin cytoskeleton via Rho family GTPases. J. Cell Biol. **150**, 567-579.

Noren, N. K., Niessen, C. M., Gumbiner, B. M., and Burridge, K. (2001). Cadherin engagement regulates Rho family GTPases. *J. Biol. Chem.* **276**, 33305-33308.

Noren, N. K., Arthur, W. T., and Burridge, K. (2003). Cadherin engagement inhibits RhoA via p190 RhoGAP. *J. Biol. Chem.* **278**, 13615-13618.

Nüsslein-Volhard, C., Wieschaus, E., and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: I. Zygotic loci on the second chromosome. *Roux's Arch. Dev. Biol.* **193**, 267-282.

Oda, H., Uemura, T., and Takeichi, M. (1997). Phenotypic analysis of null mutants for DE-cadherin and Armadillo in *Drosophila* ovaries reveals distinct aspects of their functions in cell adhesion and cytoskeletal organization. *Genes Cells* **2**, 29-40.

Ozawa, M., and Kemler, R. (1998). The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity. *J. Cell Biol.* **142**, 1605-1613.

Pacquelet A., Lin, L., and Rorth, P. (2003). Binding site for p120/δ-catenin is not required for *Drosophila* E-cadherin function in vivo. *J. Cell Biol.* **160**, 313-319.

Padash Barmchi, M., Rogers, S., and Hacker, U. (2005). *DRhoGEF2* regulates actin organization and contractility in the *Drosophila* blastoderm embryo. *J. Cell Biol.* **168**, 575-585.

Pai, L.M., Kirkpatrick, C., Blanton, J., Oda, H., Takeichi, M., Peifer, M. (1996). *Drosophila* alpha-catenin and E-cadherin bind to distinct regions of *Drosophila* Armadillo. *J. Biol. Chem.* **271**,32411-20.

Peifer, M., Orsulic, S., Sweeton, D., and Wieschaus, E. (1993). A role for the *Drosophila* segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. *Development* **118**, 1191-1207.

Perez-Moreno, M., Jamora, C., and Fuchs, E. (1993). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* **112**, 535-548.

Pettitt, J., Cox, E. A., Broadbent, I. D., Flett, A., and Hardin, J. (2003). The *Caenorhabditis elegans* p120 catenin homologue, JAC-1, modulates cadherin-cadherin function during epidermal morphogenesis. *J. Cell Biol.* **162**, 15-22.

Pokutta, S., and Weis, W.I. (2000). Structure of the dimerization and beta-catenin-binding region of alpha-catenin. *Mol. Cell* **5**, 533-43

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70,** 389-399.

Rogers, S. L., Wiedemann, U., Hacker, U., Turck, C., and Vale, R. D. (2004). *Drosophila* RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner. *Curr. Biol.* **14**, 1827-1833.

Sahai, E., and Marshall, C. J. (2002). ROCK and Dia have opposing effects on adherens junctions downstream of Rho. *Nat. Cell Biol.* **4**, 408-415.

Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., and Collard, J. G. (1999). Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J. Cell Biol.* **147,** 1009-1022.

Shibata, T., Kokubu, A., Sekine, S., Kanai, Y., and Hirohashi, S. (2004). Cytoplasmic p120ctn regulates the invasive phenotypes of E-cadherin-deficient breast cancer. *Am. J. Pathol.* **164**, 2269-2278.

Tanentzapf, G., Smith, C., McGlade, J., and Tepass., U. (2000). Apical, lateral, and basal polarization cues contribute to the development of the follicular epithelium during *Drosophila* oogenesis. *J. Cell Biol.* **151**, 891-904.

Tepass, U.Gruszynski-DeFeo, E., Haag, T. A., Omatyar, L., Torok, T., and Hartenstein, V. (1996). *shotgun* encodes *Drosophila* E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Dev.* **10**, 672-685.

Tepass, U., Truong, K., Godt, D., Ikura, M., and Peifer, M. (2001). Cadherins in embryonic and neural morphogenesis. *Nat. Rev. Mol. Cell Biol*. **1**, 91-100.

Uemura, T., Oda, H., Kraut, R., Hayashi, S., Kataoka, Y., and Takeichi, M. (1996). Zygotic *Drosophila* E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo. *Genes Dev.* **10**, 659-671.

Vaezi, A., Bauer, C., Vasioukhin, V., and Fuchs, E. (2002). Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium. *Dev. Cell* **3**, 367-381.

Wakimoto, B. T., Turner, F. R., and Kaufman, T. C. (1984). Defects in embryogenesis in mutants associated with the *antennapedia* gene complex of *Drosophila melanogaster*. *Dev. Biol.* **102**, 147-172.

Wieschaus, E. and Nüsslein-Volhard, C. (1986). Looking at embryos. *In Drosophila*, A Practical Approach. D. B. Roberts, ed. IRL Press, Oxford, England. 199-228.

Wilson, R., Vogelsang, E., and Leptin, M. (2005). FGF signaling and the mechanism of mesoderm spreading in *Drosophila* embryos. *Development* **132**, 491-501.

Xiao, K., Allison, D. F., Buckley, K. M., Kottke, M. D., Vincent, P. A., Faundez, V., and Kowalczyk, A. P. (2003). Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J. Cell Biol.* **163**, 535-545.

Yap, A. S., Niessen, C. M., and Gumbiner, B. M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J. Cell Biol.* **141**, 779-789.

Yap, A.S., and Kovacs, E.M. (2003). Direct cadherin-activated cell signaling: a view from the plasma membrane. *J. Cell Biol.* **160**, 11-16.

Fig. 3.1. Loss of *p120* slows but does not disrupt dorsal closure. Embryos. Unless noted, in all figures anterior is left. (A,B) *p120* RNAi depletes p120:GFP. GFP fluorescence, p120::GFP embryos injected with control dsRNA (A) or *p120* dsRNA (B). (C-J) Anti-phosphotyrosine. (K,L). Phalloidin. Wild-type (C,E,G,I,K). *p120* null mutants (D,F,H,J,L). (M-N) Stills, movies of representative wild-type (M) and *p120* null mutants (N), both expressing moesin-GFP. Times, lower left. (O). Mean time to complete dorsal closure±standard deviation. Scale bars=25 μm.

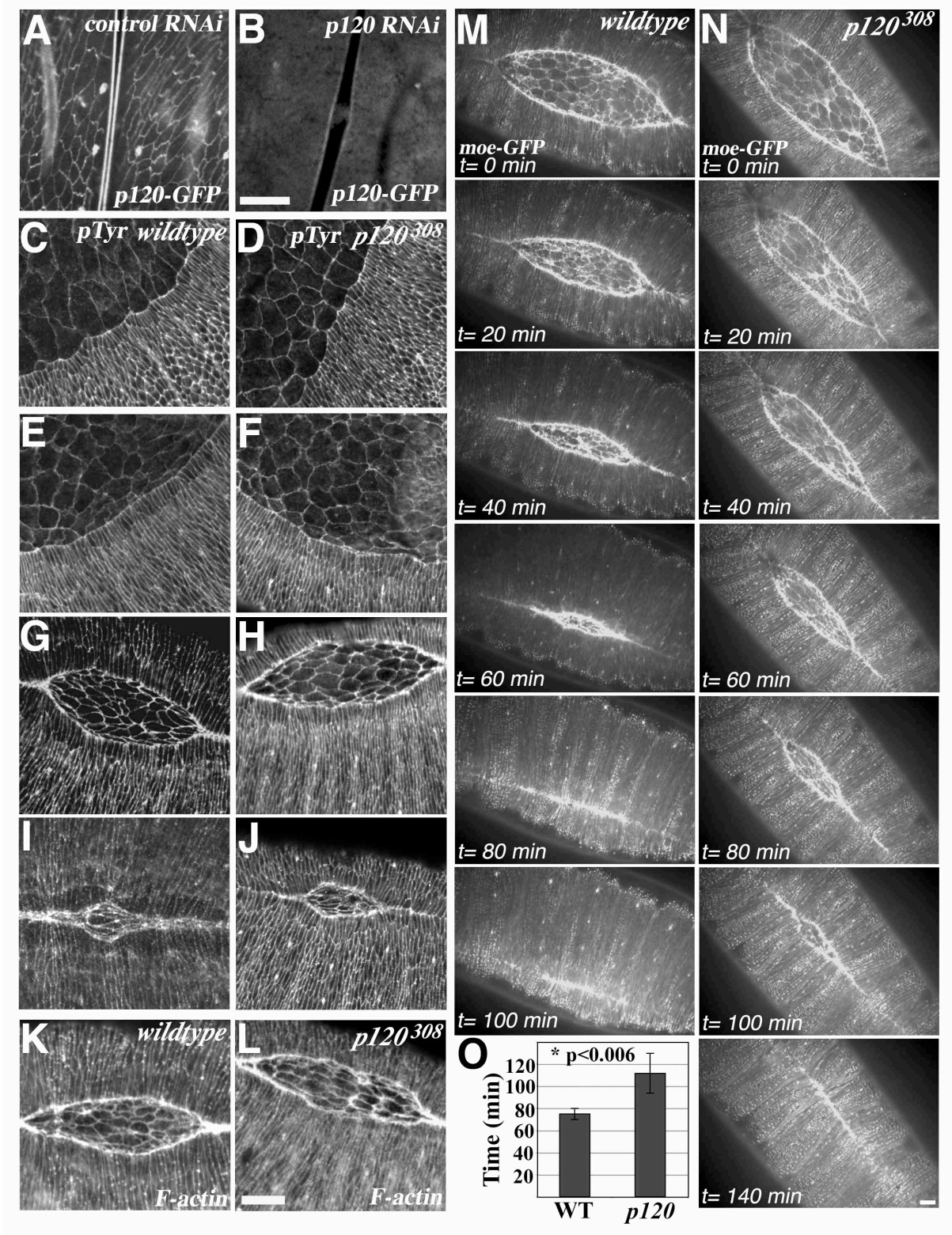

A control RNAi
p120-GFP
B p120 RNAi
p120-GFP
C pTyr wildtype
D pTyr p120308
E
F
G
H
I
J
K wildtype
F-actin
L p120308
F-actin
M wildtype
moe-GFP
t= 0 min
N p120308
moe-GFP
t= 0 min
t= 20 min
t= 20 min
t= 40 min
t= 40 min
t= 60 min
t= 60 min
t= 80 min
t= 80 min
t= 100 min
t= 100 min
t= 140 min
O
* p<0.006
Time (min)
120
100
80
60
40
20
WT
p120

Fig. 3.2. Rho1 localization during oogenesis. Egg chambers. Rho1 (red). DE-Cad (green). (A) Cross-section, stage 2-3. Rho1 accumulates laterally in follicle cells (white arrowhead) and stalk cells (red arrowhead). (B) Stage 10. Rho1 at germ cell boundaries (arrowhead). (C) DE-Cad at apical AJs of follicle cells (blue arrowhead) and in polar follicle cells (white arrow). Rho1 does not preferentially accumulate either place. (D) Cross section, stage 10B. DE-Cad and Rho1 elevated apically in centripetal follicle cells (red arrowhead). Rho1 accumulates basolaterally (blue arrowhead). (E) Grazing section basal to AJs, stage 10B follicle cells. DE-Cad is absent from tricellular junctions where Rho1 accumulates (arrows). (F-I'') Cross sections, stage 5 (F,G) and 10B (H,I). Wild-type (F,H). *p120* mutants (G,I). In both Rho1 is enriched along lateral membranes. DE-Cad is enriched at apical AJs (arrowheads). (J,K) Grazing sections, stage 10B. Rho1 at tricellular junctions. Wild-type (J). *p120* (K). (L,M) Migrating border cells. Rho1 enriched at plasma membrane and in intracellular vesicles (M, arrowhead). Scale bar=white-20 μm, red-5 μm.

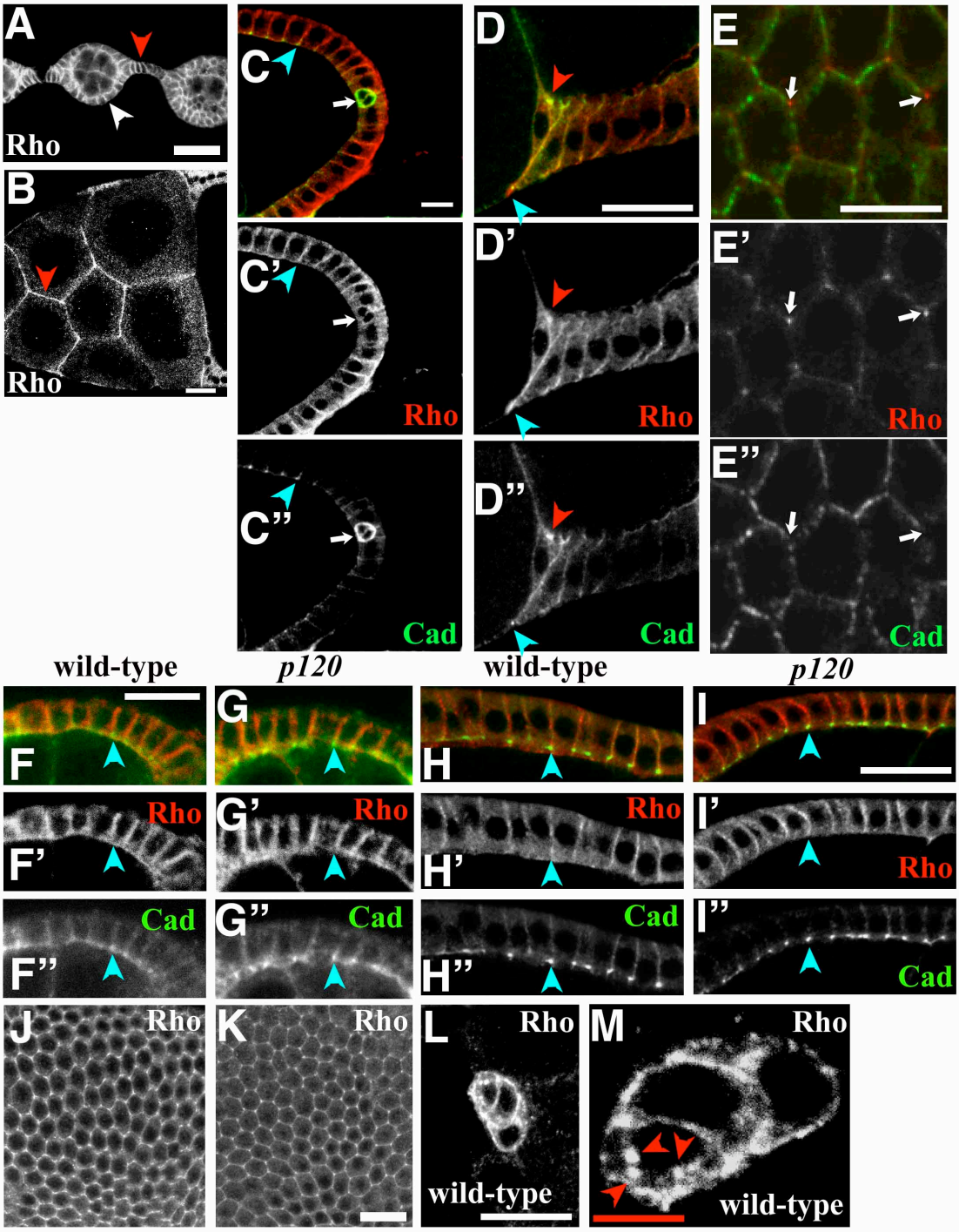

A
Rho
B
Rho
C
C'
Rho
C''
Cad
D
D'
Rho
D''
Cad
E
E'
Rho
E''
Cad
wild-type
p120
wild-type
p120
F
G
H
I
F'
Rho
G'
Rho
H'
Rho
I'
Rho
F''
Cad
G''
Cad
H''
Cad
I''
Cad
J
Rho
K
Rho
L
Rho
wild-type
M
Rho
wild-type

Fig. 3.3. Rho1 localization during embryogenesis. Rho1 (red). DE-Cad (green). (A-C) Cross sections, cellularization. Wild-type (A,B). *p120* (C). (A) Rho1 enriched at furrow canals (A, blue arrowhead). DE-Cad accumulates at basal junctions (A, yellow arrowhead) and apical AJs (A, white arrowhead). (B,C) Loss of p120 (C) does not alter Rho1 localization. (D-E) Cross-section, posterior midgut (E=close-up). Rho1 in basal puncta in invaginating midgut cells (arrowhead). DE-Cad (green, E) at sites of apical constriction. (F) Ventral furrow. DE-Cad accumulates at AJs of apically-constricting cells (arrowheads) while Rho1 does not. (G-I) Cross sections, stage 8. Rho1 accumulates where ectodermal and mesodermal cells meet (G,H, arrowheads). H=close-up of G. (I) ß-PS1 integrin. (J-L) Basolateral sections. Stages 5 (J), 9 (K), 15 (L). Rho1 cortical enrichment decreases. (M-N'') Cross sections, stage 15 hindgut. Wild-type (M). *p120* mutant (N). Rho1 accumulates basally (arrowheads). DE-Cad accumulates in apical AJs. Scale bars=20 μm.

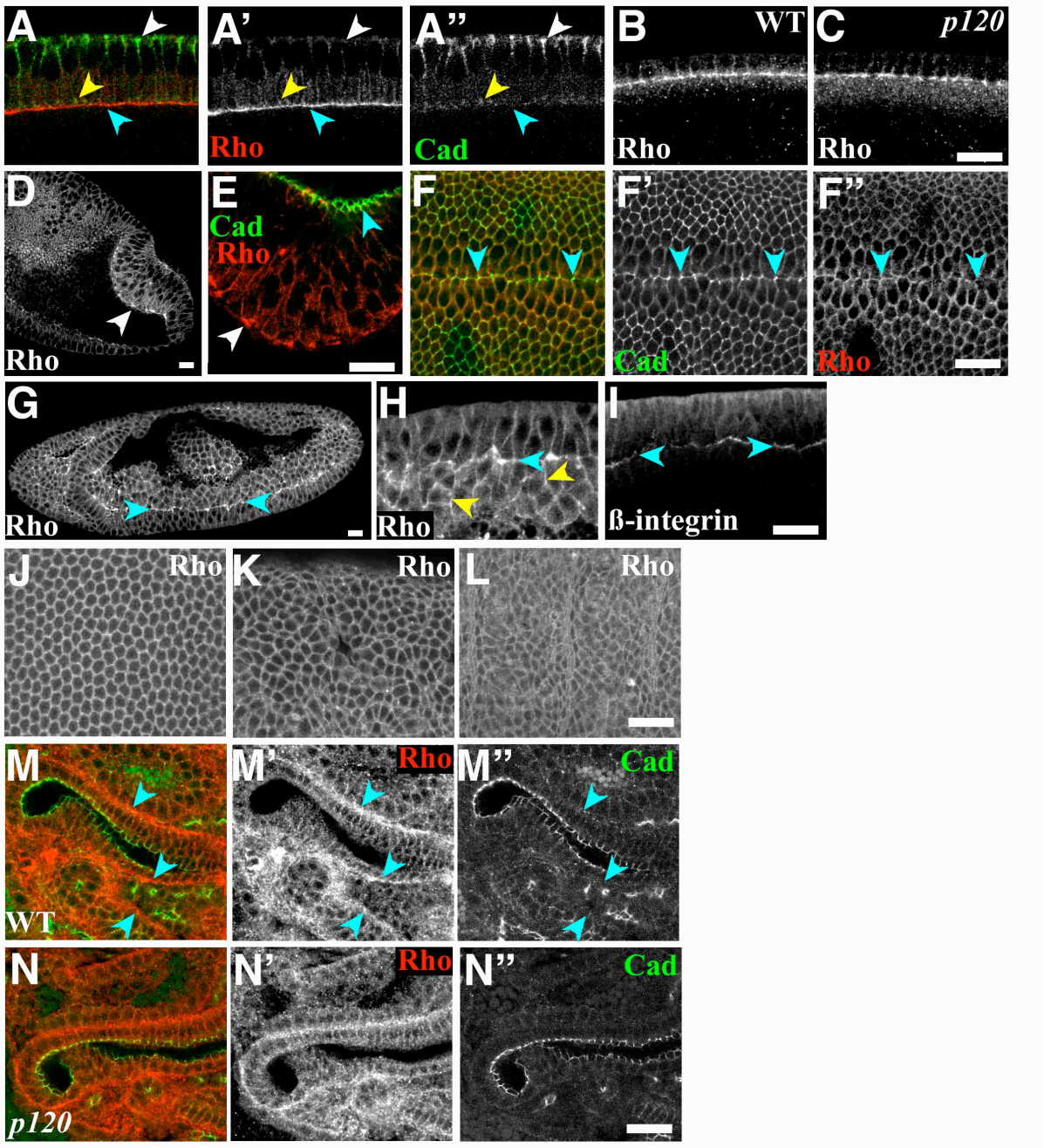

A
A'
Rho
A''
Cad
B
WT
Rho
C
p120
Rho
D
Rho
E
Cad
Rho
F
F'
Cad
F''
Rho
G
Rho
H
Rho
I
ß-integrin
J
Rho
K
Rho
L
Rho
M
WT
M'
Rho
M''
Cad
N
p120
N'
Rho
N''
Cad

Fig. 3.4. Neither p120 nor core AJ components are essential for Rho1 localization during dorsal closure. (A,B) Wild-type. DE-Cad (A,B) versus Rho1 (A',B') in apical (A,A') and basolateral (B,B') sections. (A,A') Apically, DE-Cad (A) accumulates at AJs. Leading edge (arrowhead). Rho1 (A') is at low levels in the cytoplasm and is not enriched at the leading edge (arrowhead). (B,B') Basally, DE-Cad (B) accumulates at AJs in segmental grooves (arrowhead). Rho1 (B') levels are higher and it is cortically enriched (arrowhead). (C,C') *p120* mutant. Rho1 is not elevated at the leading edge (arrowheads, C' vs. A'). (D,D') Apical RhoGEF2. Arrowheads, leading edge. (E-G) Basolateral Rho1 (arrowhead) is similar in wild-type (E), arm^{YD35} (F), or shg^{R69} zygotic mutants (G). (G') DE-Cad in a portion of G. (H,H') RhoGEF2 is enriched basolaterally and is more cortical than Rho1 (H' vs. B') at both amnioserosal (arrow) and epidermal cell (arrowhead) boundaries. (I-M'') Rho1 in shg^{R69} mutant follicle cell clones. (I-K') GFP-green, Arm-blue, DE-Cad-red. (I) Cross-section, early egg chamber. shg^{R69} mutant clone (bracket) indicated by lack of GFP. Arm accumulates at AJs at this stage despite loss of DE-Cad. (J-K') Cross-section (J-J'), grazing section (K-K'), later egg chamber. shg^{R69} mutant clone (bracket). Arm and DE-Cad are severely reduced. (L-M'') Grazing section, similar stage egg chamber as J, K. GFP-green. Rho1-blue. DE-Cad-red. (L) Rho1 accumulates normally in shg^{R69} clone. (M-M'') Closeup of L. Scale bars=white-20 μm, red-50 μm.

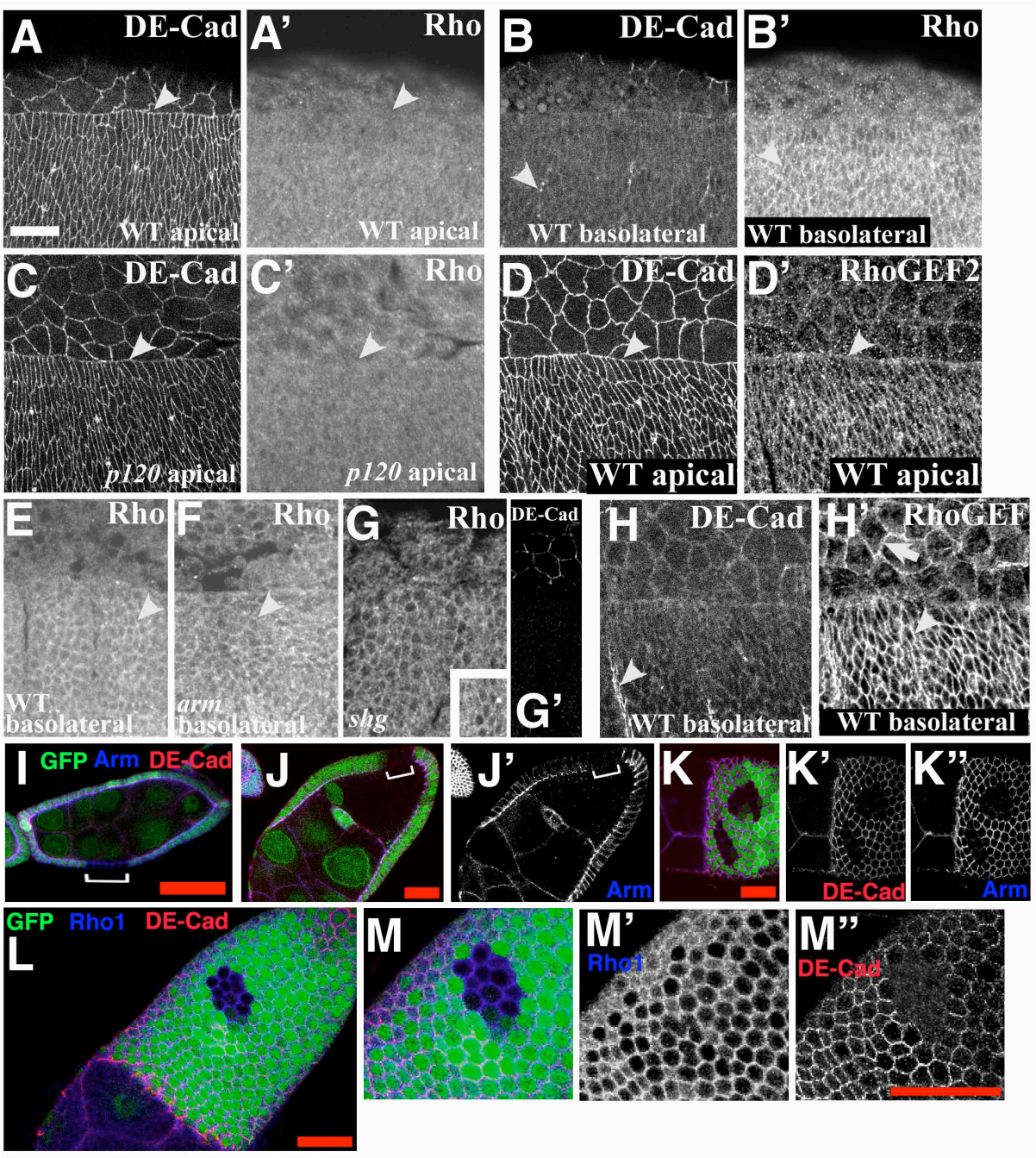

A
DE-Cad
WT apical
A'
Rho
WT apical
B
DE-Cad
WT basolateral
B'
Rho
WT basolateral
C
DE-Cad
p120 apical
C'
Rho
p120 apical
D
DE-Cad
WT apical
D'
RhoGEF2
WT apical
E
Rho
WT basolateral
F
Rho
arm basolateral
G
Rho
shg
DE-Cad
G'
H
DE-Cad
WT basolateral
H'
RhoGEF
WT basolateral
I
GFP Arm DE-Cad
J
J'
Arm
K
K'
DE-Cad
K''
Arm
GFP Rho1 DE-Cad
L
M
M'
Rho1
M''
DE-Cad

Fig. 3.5. *p120* loss-of-function does not substantially enhance or suppress *Rho1*. (A-I) Cuticle preparations, anterior up. *Rho1* (A-D), wild-type (E), or *p120 Rho1* (F-I). Embryos were divided into phenotypic classes of increasing severity (Table 3). The percentage of embryos in each class is listed below a representative cuticle. Scale bar=25 μm. (J-R) F-actin (Phalloidin). Wild-type (J,M,P). *Rho1* (K,N,Q). *p120 Rho1* (L,O,R). (J-L) Lateral view. Early dorsal closure. (M-R) Dorsal views. (M-O) Late dorsal closure. (P-R) After closure. Scale bar=20 μm.

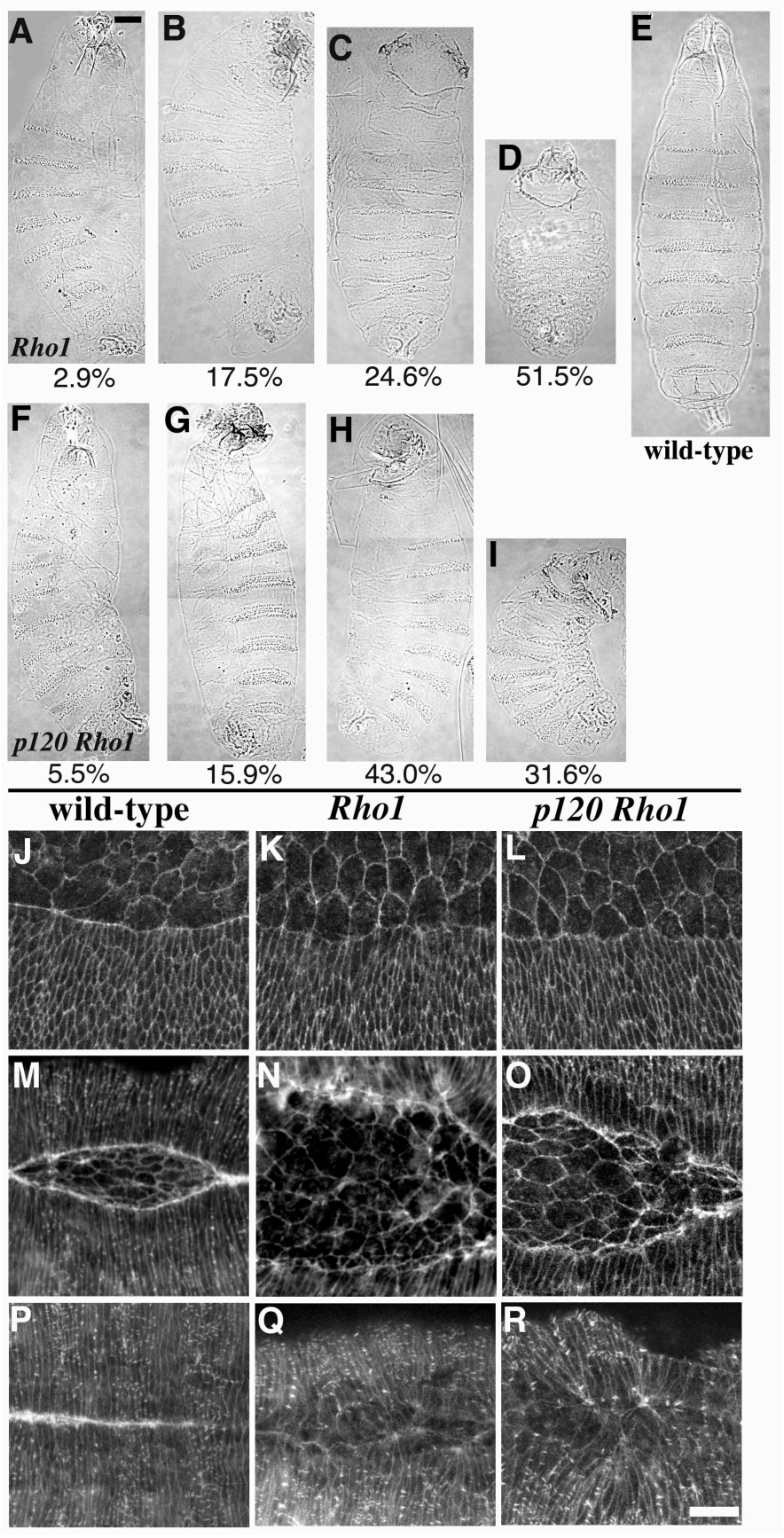

A
B
C
D
E
Rho1
2.9%
17.5%
24.6%
51.5%
wild-type
F
G
H
I
p120 Rho1
5.5%
15.9%
43.0%
31.6%
wild-type
Rho1
p120 Rho1
J
K
L
M
N
O
P
Q
R

Fig. 3.6. *Rho1* enhances a weak *shg* allele. Cuticle preparations, anterior up. (A) Wild-type. (B) shg^{G119}. Note head hole and small ventral scar. (C) *Rho1* shg^{G119}. Note head and ventral holes. (D) Schematic of shg^{G119} and shg^{2} lesions, and sequence alignment of relevant region in shg^{G119}. Gbl-cad=*Gryllus bimaculatus* (cricket) DE-cad homolog. Scale bar=25 μm.

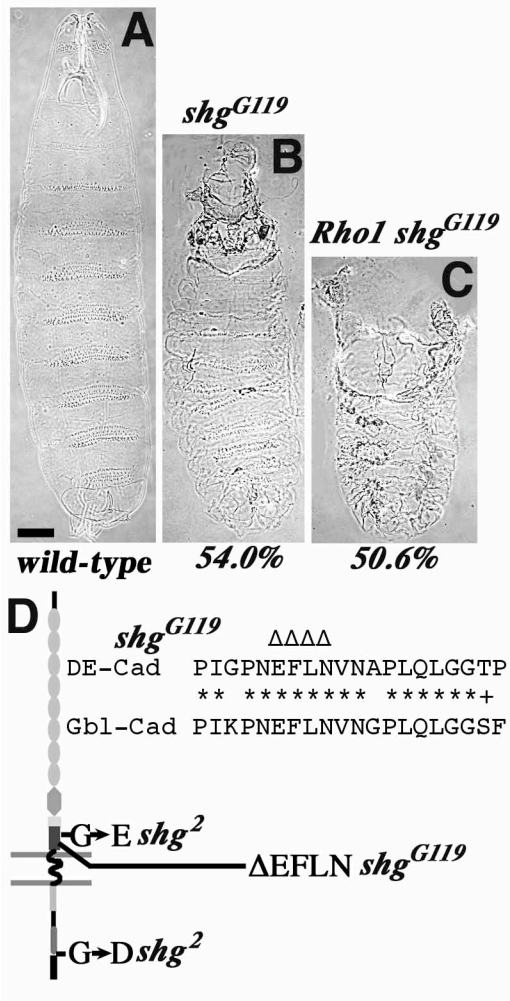

A
shgG119
B
Rho1 shgG119
C
wild-type
54.0%
50.6%
D
shgG119
ΔΔΔΔ
DE-Cad PIGPNEFLNVNAPLQLGGTP
** ******** ******+
Gbl-Cad PIKPNEFLNVNGPLQLGGSF
G→E shg2
ΔEFLN shgG119
G→D shg2

Fig. 3.7. *Rho1* suppresses both strong and null *shg* alleles. Cuticle preparations, anterior up. Major phenotypic classes (>10% of cuticles), *shg* or *Rho1 shg*. (A-D) shg^{2}. Range of defects includes ventral holes (A), fragmentary ventral cuticle (B), dorsal cuticle only (C), and dorsal cuticle with holes (D). (E,F) *Rho1* shg^{2}. >80% of embryos have ventral holes (E) or fragmentary ventral cuticle (F). (G) shg^{2} lesions in laminin G repeat and motif 5 of cadherin tail. DE-Cad versus Gbl-Cad, or mouse cadherins CAD-6 (Type II) and E-CAD (Type I). (H-J) shg^{R69}. Range of defects includes intact dorsal cuticle only (H), dorsal cuticle with holes (I), or U-shaped dorsal cuticle (J). (J-L) *Rho1* shg^{R69}. Range of defects includes fragmentary ventral cuticle (K), dorsal cuticle only (L), or dorsal cuticle with holes (M). Scale bar=25 μm.

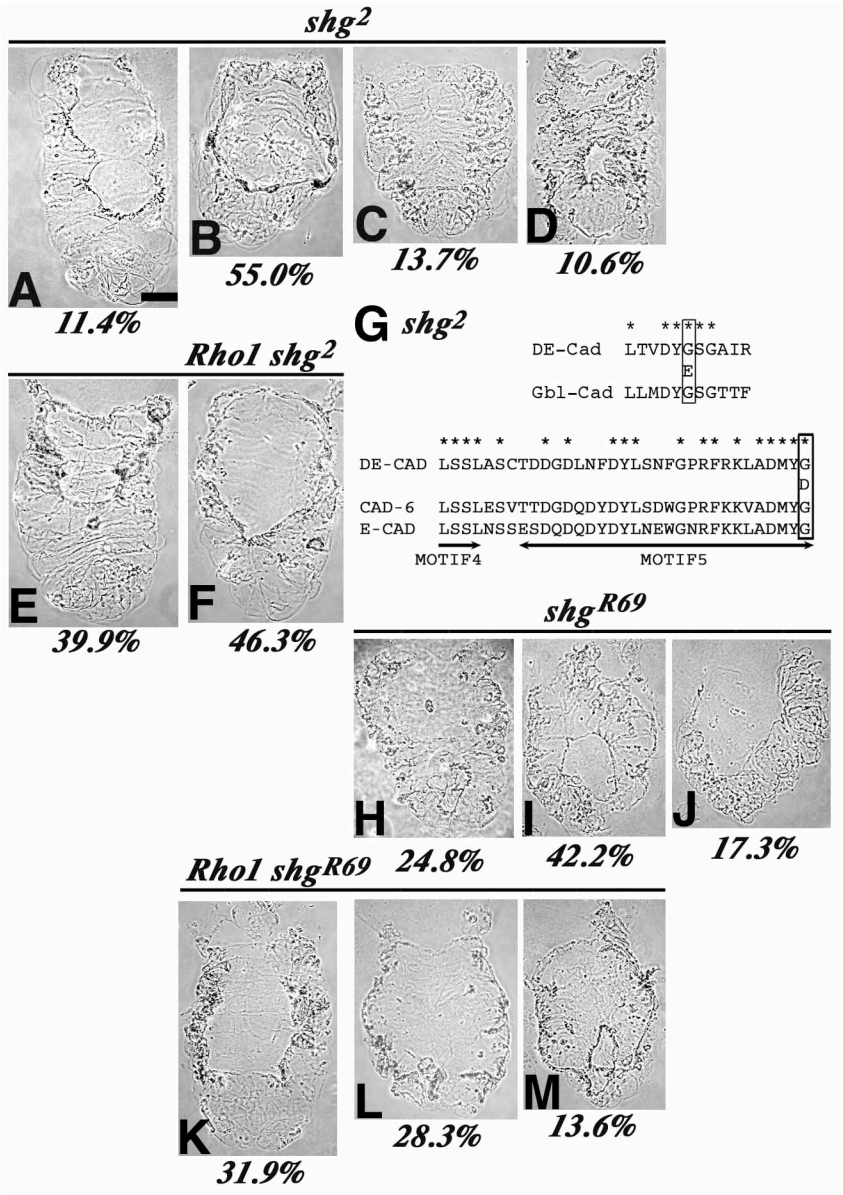

shg2
A
11.4%
B
55.0%
C
13.7%
D
10.6%
G shg2
DE-Cad LTVDYGSGAIR
Gbl-Cad LLMDYGSGTTF
Rho1 shg2
E
39.9%
F
46.3%
DE-CAD LSSLASCTDDGDLNFDYLSNFGPRFRKLADMYG
CAD-6 LSSLESVTTDGDQDYDYLSDWGPRFKKVADMYG
E-CAD LSSLNSSESDQDQDYDYLNEWGNRFKKLADMYG
MOTIF4
MOTIF5
shgR69
H
24.8%
I
42.2%
J
17.3%
Rho1 shgR69
K
31.9%
L
28.3%
M
13.6%

Fig. 3.8. Rho1 regulates DE-Cad but not Arm localization. (A,B) Wild-type (A) or *Rho1* (B) during dorsal closure. DE-Cad (red). Arm (green). Arrow-leading edge, arrowhead-amnioserosa. (C-F) DE-Cad in wild-type (C, E) and $Rho1^{1B}$ (D, F). Stage 9 (C,D; arrows-amnioserosa). Stage 17 (E,F; arrows-dorsal midline). (G) *p120 Rho1* double mutant during dorsal closure. Scale bar=20 μm.

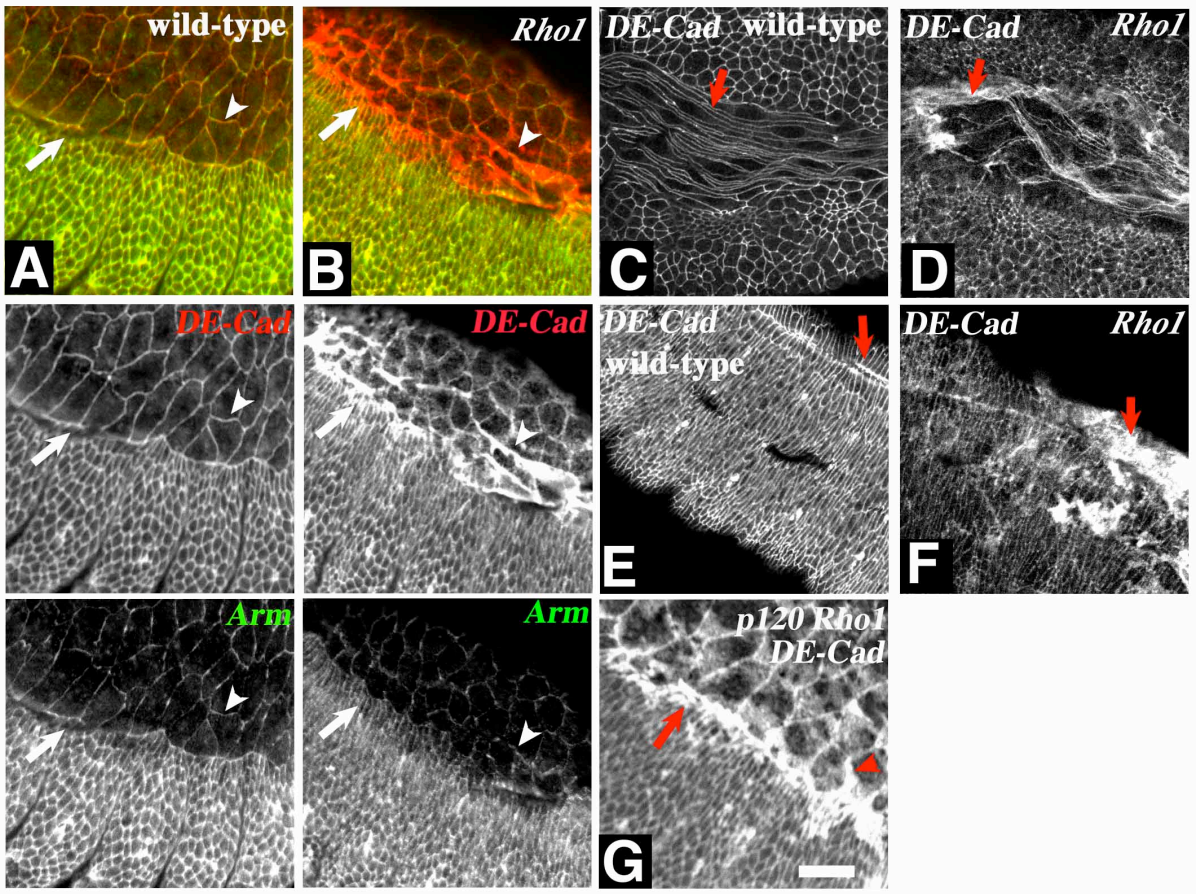

wild-type
A
Rho1
B
DE-Cad wild-type
C
DE-Cad Rho1
D
DE-Cad
DE-Cad
DE-Cad wild-type
E
DE-Cad Rho1
F
Arm
Arm
p120 Rho1
DE-Cad
G

Fig. 3.9. *p120* RNAi depletes p120:GFP. GFP fluorescence (left) and DIC images (right) of p120::GFP embryos injected with either *p120* dsRNA (A,C) or control dsRNA (B,D). (A,B) Glancing section. (C,D) Plane of focus near the embryo surface (reflection from the vitelline membrane results in a uniform background). Close-ups of these panels are in Fig. 1A,B. (A,C) No GFP fluorescence was detected in the majority of *p120 RNAi* embryos. (B,D) The majority of control dsRNA-injected embryos retain junctional p120::GFP. Scale bar = 50 μm.

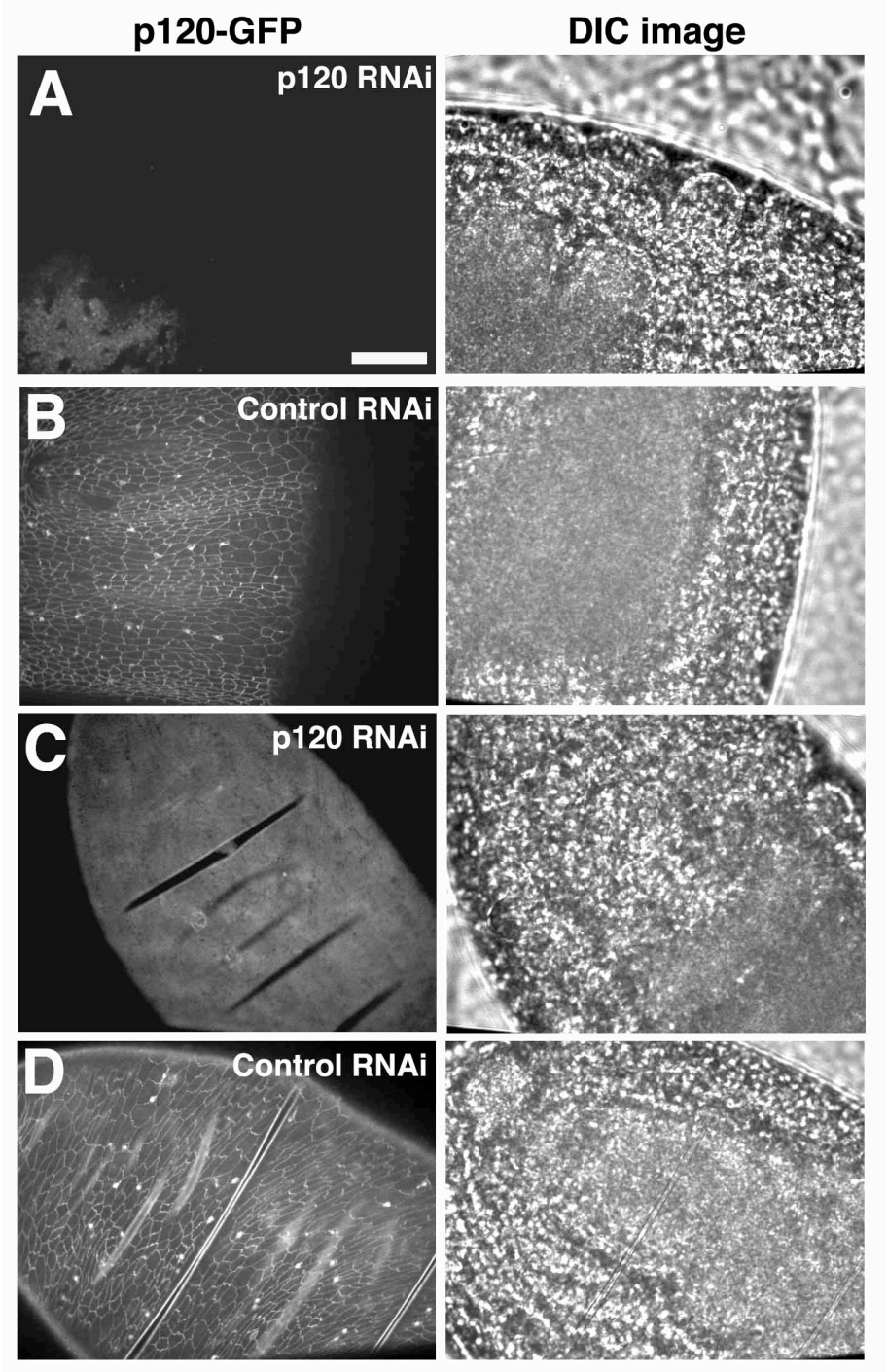

p120-GFP
DIC image
A
p120 RNAi
B
Control RNAi
C
p120 RNAi
D
Control RNAi

Table 3.1. *p120 RNAi* is not lethal

dsRNA % embryos hatched (n=)	*p120* 68.8% (655)	*gfp* 63.5% (388)	*ftz* 4.1% (393)
	Unhatched embryos-cuticle phenotypes		
Wild-type	35.7%	29.5%	0%
Head Defects	30.9%	34.6%	2.2%
Segmental fusion	8.3%	10.3%	0.7%
Tail up	7.1%	9.0%	0.7%
Disrupted	17.8%	16.7%	6.0%
ftz phenotype	0%	0%	90.2%
N	84	78	134

Table 3.2. *p120 RNAi* significantly depletes p120::GFP

DsRNA	*p120*	*Control (dys)*
Normal p120::GFP signal	10.0%	57.1%
Dim but junctional signal	3.3%	25.0%
Dim, cytoplasmic signal	3.3%	7.1%
No signal	83.3%	10.7%
N	30	28

Table 3.3. Neither loss nor over-expression of p120 substantially modifies the *Rho1* phenotype

	Phenotype	*Rho/+ x Rho/+*	*p120Rho/p120+ x p120Rho/p120+*	*Rho/+ x Rho/+*	*Rho/+; UASp120 x Rho/+; actin-GAL4*
least severe ↓	wild-type or mild head defects	2.9%	5.5%	2.2%	9.8%
	Severe head defects	17.5%	15.9%	8.6%	12.3%
	head hole with remnant head skeleton	3.4%	3.9%	5.3%	11.8%
	head hole, body >0.6 field at 20X mag.	24.6%	43.0%	33.3%	17.1%
most severe	head hole, body <0.6 field at 20X mag.	51.5%	31.6%	50.6%	48.9%
	N	1165	634	324	519

Table 3.4. *Rho1* enhances a weak *shg* allele

	Phenotype	*Rho/Cy* x *Rho/+*	*shg^{G119}/Cy* x *shg^{G119}/+*	*Rhoshg^{G119}/Cy* x *Rhoshg^{G119}/+*
Least severe	wild-type	0.8%	7.6%	0.5%
	head defect/head hole	97.6%	16.8%	17.4%
	scar in ventral cuticle	0%	50.6%	8.7%
	ventral holes	0.4%	21.7%	54.0%
	fragmentary ventral cuticle	0%	1.4%	17.4%
	dorsal cuticle only	0.4%	1.8%	1.5%
	dorsal cuticle with holes	0.4%	0%	0%
	U-shaped dorsal cuticle	0%	0%	0%
Most severe	Fragmented cuticle	0.4%	0%	0.3%
	N	255	498	378

Table 3.5. *Rho1* partially suppresses both a strong and a null *shg* allele

	Phenotype	*shg*^{2}/*Cy* x *shg*^{2}/+	*Rhoshg*^{2}/*Cy* x *Rhoshg*^{2}/+	*shg*^{R69}/*Cy* x *shg*^{R69}/+	*Rhoshg*^{R69}/*Cy* x *Rhoshg*^{R69}/+
Least severe ↓	wild-type	4.5%	5.9%	6.6%	5.1%
	head defect/head hole	1.5%	2.0%	0.8%	15.4%
	scar in ventral cuticle	0%	0%	0.3%	0.7%
	ventral holes	11.4%	39.9%	1.0%	4.0%
	fragmentary ventral cuticle	55.0%	46.3%	6.1%	31.9%
	dorsal cuticle only	13.7%	4.4%	24.8%	28.3%
	dorsal cuticle with holes	10.6%	1.4%	42.2%	13.6%
	U-shaped dorsal cuticle	2.2%	0%	17.3%	0.7%
	fragmented cuticle	0%	0%	0.5%	0%
Most severe	N	131	203	374	272

CHAPTER 4

ABELSON KINASE AND RHOGEF2 REGULATE APICAL ACTIN DURING CELL CONSTRICTION IN *DROSOPHILA*

Donald T. Fox and Mark Peifer

Preface

The following chapter contains my work on the role of Abl kinase during epithelial morphogenesis. The goal of this work was to extend the model of Abl function, involving regulation of apical actin via Ena (Grevengoed et al, 2003), from the blastoderm stages to morphogenesis. In a survey of developmental stages, I found a consistent defect on the ventral epidermis during gastrulation in *abl* mutants. This led me to characterize ventral furrow formation, a morphogenetic process involving apical cell constriction. Given the existence of other ventral furrow regulators in flies, I then examined the role of Abl in relation to other regulators. In addition to examining embryos, I took advantage of a single cell model of cell constriction (Rogers et al, 2004). Additionally, as part of this work I developed and characterized an Abl::GFP fusion to examine Abl localization. This work is currently a manuscript in preparation.

Summary

Morphogenesis involves the interaction between various cytoskeletal regulators. Investigating the interplay between cytoskeletal regulators during a given morphogenetic

event will aid in our understanding of animal development. Previous studies of ventral furrow formation, a morphogenetic event during *Drosophila* gastrulation, identified a signaling pathway involving the G-α protein Concertina (Cta) and the Rho activator RhoGEF2. While these regulators act to promote myosin accumulation and apical cell constriction, loss-of-function phenotypes for each of these pathway members is not equivalent, suggesting the existence of additional ventral furrow regulators. Here, we report the identification of Abelson kinase (Abl) as a novel ventral furrow regulator. We find that Abl kinase acts apically to suppress apical accumulation of both Enabled (Ena) and actin in mesodermal cells during ventral furrow formation. Further, we find that *RhoGEF2* mutants, but not *cta* mutants, also accumulate apical actin during ventral furrow formation. Taken together, our observations identify regulation of apical actin as a critical component that cooperates with Cta signaling during apical constriction in the *Drosophila* ventral furrow. These observations point to a conserved mechanism for Abl kinases in the regulation of actin during apical cell constriction.

Introduction

During embryonic development, many morphogenetic events shape the future body plan. These events rely on shape changes that occur at the level of single cells but must be coordinated within the context of a tissue. While developmental biologists have categorized each morphogenetic process by describing the particular cell shape changes that occur, execution of these events at a molecular level is not well understood. Most cell shape changes will require core cytoskeletal components such as actin and myosin, and thus cytoskeletal regulatory proteins are likely to dictate the type of cell shape change.

Identifying how these regulators of morphogenesis work together will aid in understanding how a group of cells execute a particular shape change.

Drosophila embryogenesis offers an attractive model to study the regulation of morphogenesis. A number of morphogenetic events during embryonic development have been characterized. These events occur in distinct cell populations and at specific times, and are likely to share mechanisms with the morphogenetic events that shape vertebrate development. Through loss of function studies, a number of mutants have been identified that affect particular processes. The formation of the ventral furrow is one such event. During gastrulation, a subset of mesodermal precursor cells along the ventral midline of the embryo apically constrict in a highly coordinated fashion. This constriction creates an invagination known as the ventral furrow that internalizes these cells as a tube; they then undergo an epithelial-mesenchymal transition. The internalization of ventral furrow cells also brings together the two rows of cells which later will form the central nervous system (CNS) midline.

Forward genetic screens identified two ventral furrow regulators that give insight into how the process works: the secreted ligand Folded Gastrulation (Fog, Wieschaus et al 1984; (Costa et al., 1994; Sweeton et al., 1991)and the G-α protein Concertina (Cta, (Parks and Wieschaus, 1991; Schupbach and Wieschaus, 1989)Loss-of-function mutations for either gene disrupt ventral furrow formation by disrupting the coordination of apical cell constriction. In these mutants, constriction occurs but in an uncoordinated fashion, disrupting tube formation (Parks and Wieschaus, 1991; Sweeton et al., 1991) Further studies established that the mesodermal transcription factors Twist and Snail specify *fog* expression in ventral furrow cells (Costa et al., 1994), and that Fog acts upstream of Concertina (Morize

et al., 1998). The G-protein coupled receptor that receives the Fog signal remains unidentified.

Next, a link between Fog/Cta signaling and the cytoskeleton was found, with the molecular identification of another regulator of the ventral furrow, the Rho1 activator RhoGEF2 (Barrett et al., 1997; Hacker and Perrimon, 1998). Epistasis experiments in embryos and cultured *Drosophila* S2 cells showed that RhoGEF2, which has a G-protein-interacting RGS domain, acts downstream of Cta (Barrett et al., 1997; Rogers et al., 2004). Downstream of RhoGEF2, the ventral furrow pathway signals through Rho1 to direct non-muscle myosin II (hereafter, myosin) accumulation. Cta, Rho1, and RhoGEF2 are sufficient for myosin accumulation and cell constriction in S2 cells (Rogers et al., 2004), and *fog*, *cta*, and *RhoGEF2* mutants exhibit defects in apical myosin localization in the ventral furrow (Dawes-Hoang et al., 2005; Nikolaidou and Barrett, 2004).

While a pathway for apical constriction signaling in the ventral furrow has been identified, loss-of-function analysis suggests that unidentified players in this process exist. While *fog* and *cta* null mutants internalize mesoderm, albeit abnormally, *RhoGEF2* null mutants exhibit a more severe phenotype, where apical constriction fails entirely and mesoderm remains on the surface of the embryo after gastrulation. This difference in phenotypic severity suggests that RhoGEF2 regulates apical constriction by both Cta-dependent and independent mechanisms. In addition to identifying other ventral furrow regulators, the role of actin in ventral furrow formation has not been closely explored.

Here, we report that Abelson kinase (Abl), plays a role during ventral furrow formation. Abl family kinases are unique among non-receptor tyrosine kinases in that they contain C-terminal actin binding domains. Mammalian Abl family members regulate actin

dynamics in a variety of cultured cell types (reviewed in (Woodring et al., 2003). Loss of both Abl and the Abl-related-gene (Arg) during mouse development disrupts actin organization during neural tube closure, resulting in developmental defects (Koleske et al., 1998). This process mirrors ventral furrow formation, as it involves apical cell constriction to create an invagination in the embryo, leading to neurectoderm internalization. Interestingly, Rho signaling has also been implicated in neural tube formation as mutants in p190RhoGAP, an Arg substrate in cultured neurons (Hernandez et al., 2004), exhibit malformed neural tubes (Brouns et al., 2000).

In *Drosophila,* Abl contributes to both axon guidance and epithelial morphogenesis. In the developing CNS, Abl negatively regulates Enabled (Gertler et al., 1995) and acts synergistically with a variety of axon guidance regulators (reviewed in (Lanier and Gertler, 2000). Among these are the Rho family GEF Trio (Forsthoefel et al., 2005; Liebl et al., 2000). Previously, we found a role for Abl during dorsal closure, a morphogenetic process whereby the embryo is enclosed in a single epithelium (Grevengoed et al., 2001). To better understand Abl's mechanism of action, we examined *abl* mutant embryos during the simple stages of blastoderm development. These studies suggested that Abl's primary mechanism of action is to negatively regulate Ena at the apical region of epithelial cells. In Abl's absence, Ena accumulates apically and has adverse effects on actin- instead of polymerizing in basal membrane furrows, actin accumulates in apical microvilli (Grevengoed et al., 2003).

Here, we describe a novel role for Abl in morphogenesis. We find that during gastrulation, Abl regulates apical constriction during mesoderm internalization. Abl's regulation of Ena appears key in this event, as Ena is down-regulated in wild-type but hyper-accumulates in *abl* mutant mesoderm. Using new localization tools, we find that Abl

concentrates at sites of apical constriction. Further, the localization of known apical constriction regulators is altered in Abl's absence, similar to embryos lacking Cta. However, unlike Cta, Abl regulates apical actin organization in both embryos and cultured S2 cells. We also find that RhoGEF2 regulates apical actin during ventral furrow formation. Importantly, *cta; abl* double mutants resemble *RhoGEF2* mutants. These results suggest that 1) an ordered apical actin network (regulated by Abl and RhoGEF2) and 2) stabilized RhoGEF2 (regulated by Cta) cooperate to direct proper myosin-based contraction. Further, this work suggests a conserved role for Abl family kinases in the regulation of apical cell constriction**.**

Materials and Methods

Abl::GFP

Using standard molecular techniques, *abl* coding sequence from cDNA clone GH09917 (Drosophila Genome Resource Center, DGRC) was fused 5' of the *egfp* start, introducing a twenty-one nucleotide linker between *abl* and *egfp* and removing the *abl* stop. For pUAS Abl::GFP, this was cloned into pUASG. For the endogenous promoter construct, two kilobases directly 5' of the *abl* start from BAC clone AC010688 (DGRC) were introduced 5' of the *abl*::GFP fusion. The resulting construct was cloned into the vector pUASG. DNA was introduced into flies via standard P-element mediated transposition. *abl*::GFP flies carrying the transgene were crossed into an abl^{1}/abl^{4} mutant background, and rescue was confirmed in adults by the absence of wild-type, non-GFP tagged Abl on a Western blot.

Fly Stocks

Mutations are described at flybase.bio.indiana.edu. $FRTRhoGEF2^{04291}$ flies were from U. Hacker (Lund University). cta^{RC10} flies were from E. Wieschaus (Princeton University). *cta*

mutant embryos were generated by crossing *cta* heterozygotes to flies carrying Df(2L)C', which lacks *cta.* *abl* and *RhoGEF2* germline clones were generated as described for *abl* in Grevengoed et al (2001). All experiments were done at 25°C. Live imaging utilized wild-type or mutant embryos expressing moesin::GFP (Edwards et al., 1997).

S2 Cells

Cell culture was as in (Rogers et al., 2002). RNAi was as in (Clemens et al., 2000). Double-stranded RNA: templates for in vitro transcription were generated by PCR with the primers encoding the T7 promoter sequence upstream of the following: *abl*-5'ACTGCATCTCCAGTTCCAGC3' and 5'ACTGCATCTCCAGTTCCAGC3', control (from pBluescript SK). Transient transfections were done using the Effectene kit (Qiagen). DNAs used were UAS-cta^{R277H}Myc, UAS-rho^{V14} (both from S. Rogers, UNC), and UAS-*abl::*GFP. Gene expression was driven from the metalothionine promoter by addition of 500μM copper sulfate to the culture medium 24 hours prior to fixation. Cells were spread on .5mg/ml ConA for 30 minutes prior to fixation.

Immunofluorescence

For myosin and RhoGEF2 staining, embryos were heat-Methanol fixed (Muller and Wieschaus, 1996). For phalloidin and Ena staining, embryos were fixed for 5 min. in 37% formaldehyde. All other fixations were in 1:1 heptane:3.7% formaldehyde for 20 minutes. Embryos were methanol-devitellinized (or hand-devitellinized for phalloidin), blocked and stained in PBS/1% goat serum/0.1%TritonX-100. S2 cells were fixed as in Rogers et al (2004). Antibodies: anti-DE-CAD2 (1:100), anti-Neurotactin (1:10), anti-β-PS1integrin (1:3), anti-Rho1 (1:100), anti-Ena (1:200), anti-Myc (1:300), anti-ArmN2 (1:200; all Developmental Studies Hybridoma Bank, DSHB), anti-Twist (1:2000; S. Roth, Max Planck

Institute), anti-Sim (1:50; S. Crews, UNC), anti-GFP (1:2000; Abcam), anti-RhoGEF2 (1:2000; S. Rogers), for Myosin, anti-Zipper (1:1250; C. Field, Harvard University), anti-Phospho-Tyrosine (1:1000, Upstate Biotech.), anti-Phospho(Y412)-c-Abl (1:250; BioSource), anti-Phospho-(Ser19)Myosin (1:200; Cell Signaling). Alexa-phalloidin (Molecular Probes) was used at 1:200. Secondary antibodies were Alexas 488, 568, and 647 (Molecular Probes). Cross-sectioning of embryos was as in Dawes-Hoang et al (2005). All samples were mounted in Aqua-Polymount (Polysciences). Fixed samples were imaged using Zeiss LSM 510 or Pascal confocal microscopes and LSM software. Live imaging used a Perkin-Elmer Ultraview spinning-disc confocal, an ORCA-ER digital camera (Hamamatsu), and Metamorph software. All images were acquired at 40X. Adobe Photoshop 7.0 was used to adjust brightness and contrast. For level comparisons, great care was taken to equally adjust compared images.

Immunoblotting

Samples were analyzed by 6% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Abl (1:100; (Grevengoed et al., 2001)and anti-Pnut (1:30; DSHB).

Results

Abl regulates apical constriction in the ventral furrow

Our previous work examining embryos prior to gastrulation established a role for Abl in regulating the type and location of actin polymerization, by regulating the localization of Ena, an actin regulator. We sought to use this mechanistic insight to return to our study of Abl's roles in morphogenesis. Abl's pre-morphogenesis roles pose a problem for this effort, since during cellularization, embryos maternally mutant for abl^4, a protein null allele

(Bennett and Hoffmann, 1992), have defects in the formation of cellularization furrows due to excess apical actin. However, we also found that the severity of these phenotypes varies with temperature: at 18°C most embryos have a large percentage of multinucleated cells due to mis-localization of actin from basal membrane furrows to apical microvilli, while at 25°C many embryos have few to no multinucleated cells (Grevengoed et al., 2003).

In this report, we took advantage of this to follow *abl* mutant embryos with weak blastoderm phenotypes into epithelial morphogenesis. This analysis revealed that *abl* mutant embryos progress through the stages of morphogenesis relatively normally until dorsal closure or germband retraction, when the majority of maternal and zygotic mutant embryos exhibit numerous morphological abnormalities (Grevengoed et al., 2001)data not shown). However, our analysis also revealed a fully penetrant defect earlier in development, specific to the ventral epidermis. We went on to characterize this ventral defect further.

Following cellularization of the blastoderm, the embryo gastrulates. This process involves two main types of cell shape changes: apical constriction that internalizes cells of the ventral furrow and posterior midgut (Sweeton et al., 1991)and cell-cell intercalation that extends the length of the germband in the anterior-posterior axis (Irvine and Wieschaus, 1994). *abl* mutant embryos show no defects in germband extension, as comparison with wild-type embryos shows similar A-P axis length at the end of gastrulation (Fig.1A vs. B). To confirm that cell-cell intercalation in the germband is unaffected by loss of *abl,* we filmed embryos expressing Moesin::GFP, which highlights the F-Actin cytoskeleton (Edwards et al., 1997). This analysis confirmed that the rearrangements of cell-cell contact that drive germband extension (Bertet et al., 2004)occur normally in *abl* mutants (Fig.1C vs. D). However, *abl* mutant embryos display consistent ventral abnormalities (Fig.1A vs. B, arrows,

Fig.1O vs. P). As this phenotype is fully penetrant, this stage of development appears to depend solely on the maternal supply of Abl. This result prompted us to investigate ventral morphogenesis in *abl* mutants in detail, focusing on the earliest morphogenetic event in these cells- the formation of the ventral furrow. We were particularly interested in this process because it closely mimics a process that fails in mice mutant for both *abl* and *arg*: neural tube closure (Koleske et al., 1998).

Ventral furrow formation is triggered by the regional expression of the transcription factor Twist, which directs cells into a mesodermal fate. Previous analysis showed that the first stage in furrow formation involves the apical constriction of individual cells located at random positions within the Twist-expressing domain. In wild-type embryos, the central constricting population of mesodermal precursor cells transition quickly from uncoordinated to coordinated apical constriction as the ventral furrow forms (Sweeton et al., 1991).

We analyzed wild-type development in detail, using surface and cross-sections, as well as time-lapse analysis of living embryos to analyze cell behavior. While the centermost cells (Fig.1E, arrow) apically constrict to adopt a triangular wedge shape (Fig.1K, arrow), the more lateral cells (Fig.1G, arrow) of the furrow do not constrict but adopt an asymmetrical morphology in order to position their apical ends towards the furrow (Fig.1K, arrowhead, (Leptin and Grunewald, 1990). Most remarkable are the outer-most furrow cells, which send their apical ends far over several neighboring cells to project towards the center of the developing furrow (Fig.1I, arrow). The difference in morphology between central constricting cells and the more lateral cells likely reflects the lack of *fog* expression in these latter mesodermal cells (Costa et al., 1994).

abl mutant embryos exhibit striking differences from wild-type soon after the onset of ventral furrow formation. In contrast to the wild-type process, which is highly coordinated, in *abl* mutants we observed uncoordinated apical constriction throughout ventral furrow formation (Fig.1F,J). While some cells and groups of cells apically constrict, other cells appear completely un-constricted. In grazing sections, these non-constricted cells appear as large, rounded cells (Fig.1F, arrowhead) next to patches of smaller, constricted cells (Fig.1F, arrow). In cross sections, the consequence of this failure to constrict on the morphology of the ventral furrow is evident. Instead of the central domain of cells constricting to a common point, constriction instead occurs in regions interspersed by non-constricted cells (Fig.1J, arrowhead). As ventral furrow formation continues, this uncoordinated constriction alters the shape of ventral furrow cells (Fig.1K vs. L), which are pulled into the furrow at varying angles (Fig.1G vs. H).

One possibility for this phenotype is that Abl is required for mesoderm specification. To examine whether the non-constricting cells in *abl* mutants are correctly specified as mesoderm, we examined expression of the mesodermal transcription factor Twist. All cells in *abl* mutant ventral furrows are Twist positive (Fig.1 M vs. N), suggesting that fate specification is not altered.

We next examined the process of ventral furrow formation in living embryos, to examine how individual cell behaviors drove the overall process. This also allowed us to address a possible caveat not ruled out our fixed embryo analysis: non-constricting cells in *abl* mutants could represent multinucleated cells from earlier blastoderm stage defects. To see if this was the case, we examined ventral furrow formation live in wild-type and *abl*

mutants. To ensure that multinucleate cells do not cause *abl* mutant defects, we only filmed *abl* mutant embryos that had no multinucleated cells at the outset (Fig.1R, t=0 min.).

Imaging of wild-type embryos revealed that mesodermal cells constrict uniformly and internalize synchronously. The difference between central and lateral cells of the furrow is evident in these movies- central cells uniformly constrict as they are internalized (Fig.1Q, t=10 min., Movie 1), whereas lateral cells do not constrict but elongate their apical ends, which orient towards the furrow as they are internalized (Fig.1Q, t=20min., Movie 1).

In contrast to wild-type, cell constriction in *abl* mutant embryos is uncoordinated, and some cells completely fail to constrict. These defects arise as soon as cell shape change initiates in the central, constricting population of ventral furrow cells (Fig.1Q vs. R, t=10 min., insets, Movie 2). When groups of neighboring cells constrict, they often appear to pull non-constricting cells neighbors toward them and often carry them inside. Other non-constricting cells persist on the surface of the embryo after the majority of cells internalize (Fig.1R, t=20, 25 min., arrow, Movie 2). These persistent cells may contribute to irregularities in the shape of ventral midline cells in many *abl* mutant embryos (Fig.1O vs. P). However, the majority of *abl* mutant embryos eventually internalize all mesoderm, allowing the two rows of midline cells to meet, as verified by localization of the midline marker single-minded (Fig.1O' vs. P'). Despite the defects in the coordination of cell constriction, those cells which do invaginate in *abl* mutants can drive formation of a furrow (with some abnormalities) in a time period roughly equivalent to wild-type (Fig.1Q vs. R, compare Movies 1 and 2).

Our use of moesin::GFP also enabled us to visualize actin dynamics in *abl* mutants during ventral furrow formation. At the onset of apical constriction in *abl* mutants, ectopic

apical patches of actin are present (Fig.1R, t=0 min., arrow). These are likely remnants of the excess microvillar actin that accumulates apically in *abl* mutants during cellularization (Grevengoed et al., 2003). This apical actin is less prominent during the course of furrow invagination. We analyze the contribution of actin localization to the *abl* ventral furrow phenotype in detail below. Taken together, these results suggest that during gastrulation, Abl regulates apical constriction in the ventral furrow but appears dispensable for the cell intercalation events of germband extension.

Abl localizes and is activated apically in embryonic epithelia

To better understand Abl's role during apical constriction, we investigated the localization of Abl during normal ventral furrow formation. Our previous mechanistic analysis of Abl function during embryogenesis (Grevengoed et al., 2003)suggested that Abl acts in the apical region of the forming cells. This observation is consistent with previous studies of Abl localization (Bennett and Hoffmann, 1992). We took two approaches to examine Abl localization and test the hypothesis that Abl is active apically. First, we used a GFP-tagging strategy previously used to study mammalian Arg::YFP in Swiss 3T3 cells (Wang et al., 2001). This strategy involved adding a C-terminal fluorescent protein tag joined by a short leader peptide (Fig.2A). We cloned this Abl::GFP fusion downstream of two kilobases of Abl 5' flanking sequence, previously shown to be sufficient for expression of a rescuing transgene (Henkemeyer et al., 1987). When introduced into flies, this Abl::GFP construct completely rescues *abl* mutants to viability and fertility, suggesting that the protein encoded by this transgene accurately replicates endogenous Abl expression and localization. As a second approach, we used a Phospho-specific antibody raised against a Tyrosine residue in the activation loop of the kinase that is specifically phosphorylated during Abl activation.

This phosphorylation site is conserved in fly Abl, and we have shown that this antibody recognizes *Drosophila* Abl in its active form (Jesse et al, in preparation). Both reagents revealed a pool of Abl that concentrates at the apical cortex of epithelial cells.

We began our analysis of Abl localization during cellularization, where we had previously hypothesized Abl to act apically. Indeed, both Abl::GFP and phospho-Abl localize apically (Fig.2 C,D,F,G,H) and cortically (Fig.2 B,E) during cellularization. At mid-cellularization, a small pool of Abl also localizes to the basal furrow canal (Fig.2G, arrow), where the majority of actin and myosin localize at this stage. Abl also overlaps adherens junctions (AJs), as shown by colocalization with DE-Cadherin (Fig.2H-H''). However, Abl also localizes more apically (Fig.2H), where other actin-associated apical proteins reside (Harris and Peifer, 2005).

Next, we examined Abl localization during gastrulation. As ventral furrow cells change shape, both total (Fig.2I, K, L) and active (Fig.2J, M, N) Abl concentrate at sites of apical constriction. Abl also localizes to sites of apical constriction in the posterior midgut during its invagination (Fig.2O). Interestingly, towards the end of ventral furrow formation, a pool of Abl concentrates basally at sites of contact between internalized mesoderm and the neighboring epithelia (Fig.2P, arrow). This may represent integrin-based contacts, as this basal population of Abl colocalizes with Beta-PS integrin (Fig.2S). We previously observed this same pool of Beta-PS integrin to colocalize with Rho1 (Fox et al., 2005). Following gastrulation, Abl::GFP and phospho-Abl strongly localize to the apical cortex of all epithelial cells (Fig.2Q, R). Thus, these new Abl localization tools revealed that Abl localizes and is active apically, and its localization and activity are elevated at sites of apical constriction.

Apical myosin fails to uniformly contract in *abl* mutants

Recent work in both embryos and S2 cells has elucidated the mechanism by which the RhoGEF2 pathway promotes cell constriction. These studies established that RhoGEF2 promotes myosin organization into an apical contractile ring in constricting cells (Nikolaidou and Barrett, 2004; Rogers et al., 2004). To understand how loss of Abl may affect this apical constriction machinery, we compared the localization of both myosin and RhoGEF2 in wild-type and *abl* mutant ventral furrows.

Myosin and RhoGEF2 largely colocalize during wild-type ventral furrow formation. During cellularization, our analysis confirmed what was previously described: myosin and RhoGEF2 localize basally to furrow canals during the progression of cellularization (Fig.3A,I, (Dawes-Hoang et al., 2005; Padash Barmchi et al., 2005; Grosshans et al., 2005). Following cellularization, most cells retain myosin in the basal yolk canal that is the remnant of the cellularization contractile apparatus. However, cells destined to form mesoderm strikingly relocalize myosin to the apical end of the cell at or just prior to the onset of constriction (Fig.3C, E, G and (Nikolaidou and Barrett, 2004). We also examined the levels of active myosin, using an antibody against the Ser-19 phosphorylation site. Cells undergoing constriction exhibit elevated levels of active myosin in the apical region (Fig. 3Q). Unlike myosin, RhoGEF2 disappears from basal furrow canals at gastrulation onset (Fig.3I; this transition occurs slightly earlier in the mesoderm), exhibiting a diffuse apical localization in all cells (Fig.3J). Next, like myosin, RhoGEF2 accumulates at the apical AJs of mesodermal precursors (Fig.3K, (Grosshans et al., 2005). Apical RhoGEF2 can be seen in cells that have not yet begun to constrict, suggesting that like myosin (Nikolaidou and Barrett, 2004), apical localization of RhoGEF2 precedes constriction. In fact, it was much

easier to identify embryos with apical RhoGEF2 prior to cell constriction, suggesting that RhoGEF2 may precede myosin to the AJ. RhoGEF2 localization during mesoderm internalization parallels both myosin and Abl localization- it accumulates strongly at sites of apical constriction in the ventral furrow (Fig.3M, O).

We next examined myosin and RhoGEF2 localization in *abl* mutants. The localization of these apical constriction regulators appears largely normal in *abl* mutants, including the basal localization of myosin in furrow canals (Fig.3B) and the apical relocalization of both myosin and RhoGEF2 in mesodermal precursors (Fig.3D, L). In cross sections, constricting cells of *abl* mutants exhibit normal localization of myosin and RhoGEF2 (Fig.3F, N, arrows), and normal levels of active (Ser-19 phosphorylated) myosin (Fig.3Q, R).

In contrast, however, in non-constricting cells of *abl* mutants, the constriction machinery localizes apically but does not assemble into an effective contractile ring. This is observed in grazing sections as diffuse apical staining in the region of the ventral furrow (Fig.3H, P, arrowheads) or in cross section as areas of faint or absent staining (Fig.3F, N, arrowheads), depending on where in a cell a particular section was cut. This *abl* phenotype contrasts that of non-constricting cells in *armadillo* mutants, which lack adherens junctions. In these mutants, the myosin network contracts without constricting the cell itself (Dawes-Hoang et al., 2005). Taken together, our results suggest that the apical constriction machinery localizes properly but fails to contract in non- constricting cells of *abl* mutants.

cta mutants resemble _abl_ mutants

During ventral furrow formation in *abl* mutants, cell constriction is uncoordinated yet mesodermal cells do internalize. This phenotype resembles the previously described

phenotypes of *cta* and *fog* mutants (Parks and Wieschaus, 1991; Sweeton et al., 1991). To understand how Abl function relates to that of other known ventral furrow regulators, we examined the *cta* phenotype in detail.

Our analysis showed striking similarities between *abl* and *cta* mutants, and also revealed new details about ventral furrow defects of *cta* mutants. Cell morphology in *cta* mutant ventral furrows closely resembles *abl* ventral furrows. In *cta* mutants, regions of constricted cells appear next to un-constricted cells (Fig.4A, C, (Parks and Wieschaus, 1991). Further, mesodermal cells are internalized at varying angles (Fig.4B) and exhibit aberrant morphology (Fig.4D). The severity of this phenotype appears comparable to *abl* mutants (compare Fig.1 to Fig.4).

We next examined the localization of the apical constriction machinery in *cta* mutants. Previously, Nikolaidou et al (2004) reported a lower level of apical myosin in constricting cells in *cta* mutants than in wild-type. However, when we imaged wild-type and mutant embryos stained and imaged together, we saw no decrease in apical myosin levels in constricting cells relative to wild-type (Fig.4G vs. Fig.4H). We should note that we did find sections with little or no apical myosin, but suspect these are sections that passed through few or no constricting cells. Similar to *abl* mutants, myosin failed to assemble into contractile structures in non-constricting cells of *cta* mutants (Fig.4E). As in *abl* mutants, RhoGEF2 concentrates only at sites where cells successfully underwent apical constriction (Fig.4F, I vs. J), consistent with the idea that the failure of myosin contraction in *cta* mutants is RhoGEF2-dependent. Thus, both *abl* and *cta* mutants exhibit regions of non-constricted cells where RhoGEF2 and myosin fail to assemble a contractile network.

Abl promotes Actin accumulation while Cta promotes Myosin accumulation in S2 cells

Our loss of function studies suggested that both Abl and Cta promote myosin-based contraction during ventral furrow formation. To better understand how these two proteins function in this process, we took advantage of a single cell assay for myosin organization during cell constriction in S2 cells. Previously, overexpression of RhoGEF2 pathway components including active forms of Cta and Rho were shown to promote myosin accumulation in these cells (Rogers et al., 2004). Overexpression of constitutive active Rho^{V14} (Paterson et al., 1990) also promotes actin accumulation (S. Rogers, pers. comm.). We thus examined both myosin and actin localization in S2 cells in response to Abl, Cta, and Rho overexpression. Additionally, we used the accessibility of the S2 cell system in both overexpression through transfection and loss of function through RNAi to test whether Abl may function in the RhoGEF2 signaling pathway. To overexpress Abl, we cloned the Abl::GFP construct lacking the promoter region into a UAS expression vector (Fig.5A). We then drove expression of this construct using metalothionine-Gal4.

Expression of Abl in S2 cells results in increased overall tyrosine kinase activity, as assessed by Phospho-Tyrosine levels (Fig.5B-B'). In roughly one half of these cells, the morphology changes from a smooth, continuous peripheral edge to one containing numerous projections (Fig.5C). Further, these UAS-Abl cells contain increased levels of peripheral actin (Fig.5C', seen in 23/50 cells). In contrast, these cells exhibit normal levels of P(Ser19)myosin (no change in 45/50 cells, Fig.5C''').

Overexpression of Cta in S2 cells produces a very different result from Abl-overexpression. As in Rogers et al (2004), we saw that myosin levels increase in response to overexpressed active (R277H, (Morize et al., 1998) Cta::Myc (Fig.5D'', seen in 30/50 cells).

In contrast, these cells do not exhibit a consistent increase in actin levels (Fig.5D', no change in 43/50 cells). Thus, Abl and Cta appear to promote actin and myosin accumulation, respectively, in S2 cells.

Overexpression of Rho^{V14} results in an increase of both actin (Fig.5E', seen in 37/50 cells) and Phospho-(Ser19)myosin (Fig.5E'', seen in 49/50 cells) levels in S2 cells. Unlike in UAS-Abl cells, the actin accumulation in Rho^{V14} cells localizes to a ring above the cell center where it colocalizes with myosin (Fig.5E'''). Myosin localization in Rho^{V14} cells differs qualitatively from most Cta^{R277H} cells in that it localizes into a smaller ring-like structure (Fig.5E'' vs. Fig.5D''), as was previously observed in constricting S2 cells (Rogers et al., 2004). Thus, Rho overexpression combines elements of both Abl and Cta overexpression by concentrating increased levels of actin and active myosin into a ring-like contractile structure.

We next asked whether Abl acts in concert with Rho during S2 cell constriction. Specifically, we asked whether Abl is required for the Rho gain-of-function phenotype by performing control and *abl* RNAi in Rho^{V14} cells. Despite efficient knockdown of Abl protein (Fig.5), the actin and Phospho-(Ser19)Myosin Rho overexpression phenotypes are unaffected in *abl* RNAi cells relative to controls (Fig.5F''' vs. Fig.5G'''). This result suggests that Abl does not act downstream of Rho signaling in S2 cells.

Abl regulates Enabled and Actin during ventral furrow formation

Our results from overexpression in cultured cells combined with the earlier work on Abl as an actin regulator in both flies and mammalian cells suggested that Abl might regulate myosin-based constriction during ventral furrow formation via its effects on actin. To test this hypothesis, we examined whether Abl regulates Ena and thus actin localization during

ventral furrow formation. Prior to apical constriction, actin localizes predominantly to basal furrow canals, while Ena has no specific localization pattern (Fig.6A-A''; (Grevengoed et al., 2003). As ventral furrow formation begins, actin relocalizes apically in ventral furrow cells and disappears from its basal location (Fig.6B, D, arrow), similar to myosin (Fig.3). At this time, a distinguishable pool of Ena begins to localize at apical AJs in most cells (Fig.6C, arrow). However, Ena remains noticeably absent from cells of the ventral furrow as apical constriction initiates (Fig.6C, arrowhead).

Loss of Abl disrupts both Ena and actin localization during ventral furrow formation. Prior to cell constriction, *abl* mutants exhibit a considerable amount of ectopic apical actin that colocalizes with ectopic apical Ena (Fig.6E-E''; (Grevengoed et al., 2003). This actin localization mirrors the patches of F-Actin seen prior to constriction in our live analysis (Fig.1, t=0min., Movie 2). The ectopic apical actin persists throughout the embryo as ventral furrow formation begins (Fig.6F). As gastrulation proceeds in mutant embryos, actin persists apically but is also present along the lateral membranes of all mesodermal cells, in contrast to the highly apically polarized distribution of actin in wild-type (Fig.6D vs. Fig.6H). Further, apical Ena levels are elevated in *abl* mutants (Fig.6C vs. Fig.6G). Importantly, Ena accumulates in constricting cells of *abl* mutants- the same cells that exhibit no detectable Ena localization in wild-type at this stage (arrowheads in Fig.6C vs. Fig.6G). In summary, Abl promotes an enhanced down-regulation of Ena in the ventral furrow cells, allowing for apically polarized actin accumulation in constricting cells.

As the mis-localization of both actin and Ena contributes to other *abl* phenotypes, we next examined whether mis-localization of Ena during ventral furrow formation is causative in the *abl* ventral furrow phenotype. We did so by genetically reducing the dose of Ena in an

abl mutant background and asked if this rescues the *abl* ventral furrow phenotype. In embryos from maternally ena^{210}/+; *abl* females, the ventral furrow phenotype is largely suppressed. In most of these embryos, apical constriction and mesoderm internalization appeared normal (Fig.6R). Taken together, these results suggest that while Abl negatively regulates apical Ena and thus actin localization in all cells, an enhanced Abl-dependent downregulation of Ena allows for ordered apical actin during ventral furrow formation.

RhoGEF2, but not Cta, regulates Actin localization in the ventral furrow

Our results suggest that in addition to regulation of myosin, regulation of actin is required for ordered apical constriction in the ventral furrow. We next asked whether other ventral furrow regulators also direct actin localization during this process, by examining actin and Ena localization in *cta* and *RhoGEF2* mutant embryos. As the *abl* actin phenotype arises prior to gastrulation, we examined both cellularization and gastrulation stages.

cta mutants show no defects in actin localization during either cellularization (Fig.6I) or gastrulation (Fig.6J,L). These results parallel our S2 cell results, where Cta overexpression has no effect on actin levels (Fig.5). Further, downregulation of Ena occurs normally in *cta* mutant furrows (Fig.6I, K). Thus, while *abl* and *cta* mutants exhibit similar ventral furrow phenotypes, these proteins appear to regulate this process via distinct mechanisms.

In contrast to *cta* mutants, *RhoGEF2* mutants display an actin phenotype very similar to *abl* mutants. During *RhoGEF2* mutant cellularization, ectopic apical actin accumulates, as in *abl* mutants (Fig.6E vs. Fig.6M; previously reported by Padash-Barmchi et al, 2005). However, Ena does not accumulate apically at these stages as is seen in *abl* mutants (Fig.6E' vs. Fig.6M'). Ectopic apical actin remains in all cells during ventral furrow formation, similar to *abl* mutants (Fig.6F vs. Fig.6N). However, unlike in *abl* mutants, Ena is properly

down-regulated in *RhoGEF2* mutant mesoderm (Fig.6G vs. Fig.6O), which remains on the surface in these embryos. In later *RhoGEF2* mutants, actin and Ena localize to a distinct apical domain (Fig.6Q-Q''), a defect not seen in *abl* mutants. Thus, both Abl and RhoGEF2 regulate apical actin organization prior to and during ventral furrow formation. While Abl acts via Ena, RhoGEF2 appears to act via a distinct mechanism.

Discussion

Epithelial morphogenesis involves the cooperation of numerous cytoskeletal regulators. Here, we identify Abl kinase as an additional input into apical constriction in the *Drosophila* ventral furrow. Our results suggest that in addition to the known signaling pathway that promotes apical myosin activation, regulation of apical actin by Abl and RhoGEF2 is also a critical part of this process. These results highlight the likely conservation of Abl and Rho signaling in apical constriction.

Actin regulation is a key component of mesoderm internalization

Previous work established myosin as a key output of the RhoGEF2 signaling pathway during mesoderm internalization (Dawes-Hoang et al, 2005; Nikolaidou et al, 2004; Rogers et al, 2004). However, ambiguities remained regarding the circuitry of this pathway, as *RhoGEF2* mutants are more severe than *cta* or *fog* mutants. Here, we suggest that RhoGEF2's earlier role in actin organization, during cellularization, accounts for the difference in phenotype.

Figure 7 presents a model for the roles of each ventral furrow regulator examined in our study. This model accounts for the role of each regulator prior to and during apical cell constriction. Prior to gastrulation, apical Abl and basal RhoGEF2 act to maintain actin in

basal furrow canals (Fig. 7A). In both *abl* (Fig. 6E; Grevengoed et al, 2003) and *RhoGEF2* mutants (Fig. 6M; Padash-Barmchi et al, 2005), ectopic actin accumulates apically (Fig. 7B, D). As Fog signaling is not active at this stage, loss of Cta has no effect (Fig. 6I, Fig. 7C).

During gastrulation, two components cooperate to ensure proper mesodermal constriction: 1) stabilized apical RhoGEF2, achieved through Fog/Cta signaling, and 2) organized apical actin, mediated by Abl and RhoGEF2 (Fig. 7E). In an *abl* mutant (Fig. 7F), Fog/Cta signaling is present, allowing for RhoGEF2 stabilization and myosin contraction. However, due to ectopic actin (Fig. 6F), via Ena's inappropriate accumulation (Fig. 6G), this signal is not sufficient to allow for myosin contraction in all cells (Fig. 3F). In a *cta* mutant (Fig. 7G), lack of a stabilizing signal for RhoGEF2 prevents stabilized apical myosin. Thus, the apical constriction machinery is less stable, resulting in some un-constricted cells (Fig. 4H). In a *RhoGEF2* mutant, both components are absent: the lack of RhoGEF2 prevents apical myosin stabilization (Dawes-Hoang et al, 2005; Nikolaidou et al, 2004), but in addition, ectopic apical actin (Fig. 6N) completely eliminates cell constriction in *RhoGEF2* mutants (Fig.7H).

Our model points to organized apical actin as an important target of apical constriction regulators. While we find that both Abl and RhoGEF2 regulate apical actin, our data argues that each regulator functions via a distinct mechanism. First, this phenotype arises during cellularization, where Abl and RhoGEF2 have distinct, non-overlapping localization patterns (Fig. 2H vs. Fig. 3I). Second, while Abl clearly acts through Ena, loss of RhoGEF2 disrupts actin without altering Ena localization (Fig. 6M'). Finally, Abl does not behave as a Rho effector in S2 cells (Fig. 5H).

Unanswered questions remain regarding regulation of the cytoskeleton during ventral furrow formation. Our data points to Ena as in important factor in cytoskeletal regulation in the mesoderm. While Ena accumulates outside of the mesoderm during gastrulation, it remains down-regulated in the ventral furrow. This persistent down-regulation could be due to increased Abl activity or could result from the transcriptional re-programming that directs the epithelial-mesenchymal transition in these cells. Another unexplored aspect of this process is the disappearance of both actin and myosin from the base of the constricting cells. This process occurs normally in all mutants examined here, including *RhoGEF2* mutants. The future identification of basal ventral furrow cues will address whether the loss of basal actin and myosin is required for constriction and internalization, an important issue regarding this type of cell shape change.

Abl is required for a specific type of cell shape change

Our examination of the role of Abl during gastrulation lends evidence to the idea that different cytoskeletal regulators direct distinct morphogenetic processes. Abl is required for coordinated apical constriction in the ventral (Fig. 1F), but is dispensable for cell-cell intercalation in the germband (Fig. 1D). This phenotype mirrors that of *fog* mutants, which have defects in ventral furrow formation (Sweeton et al, 1991) but not in germband extension (Bertet et al, 2004). Thus, while both processes require a dynamic reorganization of myosin (Nikolaidou et al, 2004; Zallen and Wieschaus, 2004), distinct regulators act in each process.

However, the picture becomes more complex when considering other known roles for ventral furrow regulators outside of the mesoderm. While Fog, Cta, and RhoGEF2 are required for apical constriction in the posterior midgut and salivary glands (Nikolaidou et al, 2004; Barrett et al, 1997; Sweeton et al, 1991), Abl appears dispensable for these events (data

not shown). This may account for the fact that loss of Fog or Cta more closely resembles loss of RhoGEF2 in the posterior midgut. Thus, certain types of apical constriction may require different levels of regulation. In the future, it will be interesting to explore the role of Fog, Cta, and RhoGEF2 during dorsal closure, an Abl-dependent morphogenetic event (Grevengoed et al, 2001).

A conserved role for Abl and Rho signaling during apical constriction

This study also suggests a mechanistic connection between the fly process of ventral furrow formation and the vertebrate process or neural tube closure. Both processes involve actin-based apical constriction to internalize sheet of cells into a tube-like structure. Importantly, knockout of the two Abl family kinases in mice results in disorganized actin and defects in neural tube closure (Kokeske et al, 1998). Further, mammalian Ena (Mena) also plays a role in neural tube closure, in conjunction with Profilin (Lanier et al, 1999). Thus, Abl/Ena signaling may represent a conserved mechanism of actin regulation during apical constriction.

Rho signaling also plays a role in neural tube closure. Mice lacking the Rho regulator p190 RhoGAP exhibit neural tube defects associated with ectopic F-Actin and a lack of apical constriction (Brouns et al, 2000). Interestingly, p190 RhoGAP is a substrate of Arg in the mouse brain (Hernandez et al, 2004), suggesting a possible direct link between Abl and Rho signaling in apical constriction. The role of *Drosophila* p190 RhoGAP in the ventral furrow has yet to be examined directly, however the putative RhoGAP68F was recently implicated in ventral furrow formation (Sanny et al, 2006). It is likely that work in both fly and vertebrate systems will aid in understanding conserved mechanisms of apical cell constriction.

Drosophila Abl localization: new tools and insights

This study also makes use of new localization tools to understand the role of *Drosophila* Abl. Our initial studies using these tools confirmed what we had hypothesized previously- that Abl concentrates its activity apically in epithelial cells (Grevengoed et al, 2003). Future use of these tools will enhance our understanding of Abl function during morphogenesis. Use of Abl::GFP will allow for future time-lapse studies of Abl dynamics. This will be interesting to observe in the context of processes that involve dynamic cytoskeletal rearrangements. Additionally, Phospho-Abl localization may pinpoint subsets of total Abl that are active. Such observations may identify other developmental processes and sub-cellular locations where Abl plays a role.

References

Barrett, K., Leptin, M. and Settleman, J. (1997). The Rho GTPase and a putative RhoGEF mediate a signaling pathway for the cell shape changes in Drosophila gastrulation. *Cell* **91**, 905-15.

Bennett, R. L. and Hoffmann, F. M. (1992). Increased levels of the Drosophila Abelson tyrosine kinase in nerves and muscles: subcellular localization and mutant phenotypes imply a role in cell-cell interactions. *Development* **116**, 953-66.

Bertet, C., Sulak, L. and Lecuit, T. (2004). Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation. *Nature* **429**, 667-71.

Brouns, M. R., Matheson, S. F., Hu, K. Q., Delalle, I., Caviness, V. S., Silver, J.,

Bronson, R. T. and Settleman, J. (2000). The adhesion signaling molecule p190 RhoGAP is required for morphogenetic processes in neural development. *Development* **127**, 4891-903.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A* **97**, 6499-503.

Costa, M., Wilson, E. T. and Wieschaus, E. (1994). A putative cell signal encoded by the folded gastrulation gene coordinates cell shape changes during Drosophila gastrulation. *Cell* **76**, 1075-89.

Dawes-Hoang, R. E., Parmar, K. M., Christiansen, A. E., Phelps, C. B., Brand, A. H. and Wieschaus, E. F. (2005). folded gastrulation, cell shape change and the control of myosin localization. *Development* **132**, 4165-78.

Edwards, K. A., Demsky, M., Montague, R. A., Weymouth, N. and Kiehart, D. P. (1997). GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in Drosophila. *Dev Biol* **191**, 103-17.

Forsthoefel, D. J., Liebl, E. C., Kolodziej, P. A. and Seeger, M. A. (2005). The Abelson tyrosine kinase, the Trio GEF and Enabled interact with the Netrin receptor Frazzled in Drosophila. *Development* **132**, 1983-94.

Fox, D. T., Homem, C. C., Myster, S. H., Wang, F., Bain, E. E. and Peifer, M. (2005). Rho1 regulates Drosophila adherens junctions independently of p120ctn. *Development* **132**, 4819-31.

Gertler, F. B., Comer, A. R., Juang, J. L., Ahern, S. M., Clark, M. J., Liebl, E. C. and Hoffmann, F. M. (1995). enabled, a dosage-sensitive suppressor of mutations in the Drosophila Abl tyrosine kinase, encodes an Abl substrate with SH3 domain-binding properties. *Genes Dev* **9**, 521-33.

Grevengoed, E. E., Fox, D. T., Gates, J. and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. *J Cell Biol* **163**, 1267-79.

Grevengoed, E. E., Loureiro, J. J., Jesse, T. L. and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in Drosophila. *J Cell Biol* **155**, 1185-98.

Grosshans, J., Wenzl, C., Herz, H. M., Bartoszewski, S., Schnorrer, F., Vogt, N., Schwarz, H. and Muller, H. A. (2005). RhoGEF2 and the formin Dia control the formation of the furrow canal by directed actin assembly during Drosophila cellularisation. *Development* **132**, 1009-20.

Hacker, U. and Perrimon, N. (1998). DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in Drosophila. *Genes Dev* **12**, 274-84.

Harris, T. J. and Peifer, M. (2005). The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. *J Cell Biol* **170**, 813-23.

Henkemeyer, M. J., Gertler, F. B., Goodman, W. and Hoffmann, F. M. (1987). The Drosophila Abelson proto-oncogene homolog: identification of mutant alleles that have pleiotropic effects late in development. *Cell* **51**, 821-8.

Hernandez, S. E., Settleman, J. and Koleske, A. J. (2004). Adhesion-dependent regulation of p190RhoGAP in the developing brain by the Abl-related gene tyrosine kinase. *Curr Biol* **14**, 691-6.

Irvine, K. D. and Wieschaus, E. (1994). Cell intercalation during Drosophila germband extension and its regulation by pair-rule segmentation genes. *Development* **120**, 827-41.

Koleske, A. J., Gifford, A. M., Scott, M. L., Nee, M., Bronson, R. T., Miczek, K. A. and Baltimore, D. (1998). Essential roles for the Abl and Arg tyrosine kinases in neurulation. *Neuron* **21**, 1259-72.

Lanier, L. M. and Gertler, F. B. (2000). From Abl to actin: Abl tyrosine kinase and associated proteins in growth cone motility. *Curr Opin Neurobiol* **10**, 80-7.

Lanier LM, Gates MA, Witke W, Menzies AS, Wehman AM, Macklis JD, Kwiatkowski D, Soriano P, Gertler FB. (1999). Mena is required for neurulation and commissure formation. *Neuron* **22,** 313-25.

Leptin, M. and Grunewald, B. (1990). Cell shape changes during gastrulation in Drosophila. *Development* **110**, 73-84.

Liebl, E. C., Forsthoefel, D. J., Franco, L. S., Sample, S. H., Hess, J. E., Cowger, J. A., Chandler, M. P., Shupert, A. M. and Seeger, M. A. (2000). Dosage-sensitive, reciprocal

genetic interactions between the Abl tyrosine kinase and the putative GEF trio reveal trio's role in axon pathfinding. *Neuron* **26**, 107-18.

Morize, P., Christiansen, A. E., Costa, M., Parks, S. and Wieschaus, E. (1998). Hyperactivation of the folded gastrulation pathway induces specific cell shape changes. *Development* **125**, 589-97.

Muller, H. A. and Wieschaus, E. (1996). armadillo, bazooka, and stardust are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in Drosophila. *J Cell Biol* **134**, 149-63.

Nikolaidou, K. K. and Barrett, K. (2004). A Rho GTPase signaling pathway is used reiteratively in epithelial folding and potentially selects the outcome of Rho activation. *Curr Biol* **14**, 1822-6.

Padash Barmchi, M., Rogers, S. and Hacker, U. (2005). DRhoGEF2 regulates actin organization and contractility in the Drosophila blastoderm embryo. *J Cell Biol* **168**, 575-85.

Parks, S. and Wieschaus, E. (1991). The Drosophila gastrulation gene concertina encodes a G alpha-like protein. *Cell* **64**, 447-58.

Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K. and Hall, A. (1990). Microinjection of recombinant p21rho induces rapid changes in cell morphology. *J Cell Biol* **111**, 1001-7.

Rogers, S. L., Rogers, G. C., Sharp, D. J. and Vale, R. D. (2002). Drosophila EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. *J Cell Biol* **158**, 873-84.

Rogers, S. L., Wiedemann, U., Hacker, U., Turck, C. and Vale, R. D. (2004). Drosophila RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner. *Curr Biol* **14**, 1827-33.

Sanny, J., Chui, V., Langmann, C., Pereira, C., Zahedi, B., and Harden, N. (2006). *Drosophila* RhoGAP68F is a putative GTPase activating protein for RhoA participating in gastrulation. *Dev. Genes Evol.* Apr 12; (Epub ahead of print).

Schupbach, T. and Wieschaus, E. (1989). Female sterile mutations on the second chromosome of Drosophila melanogaster. I. Maternal effect mutations. *Genetics* **121**, 101-17.

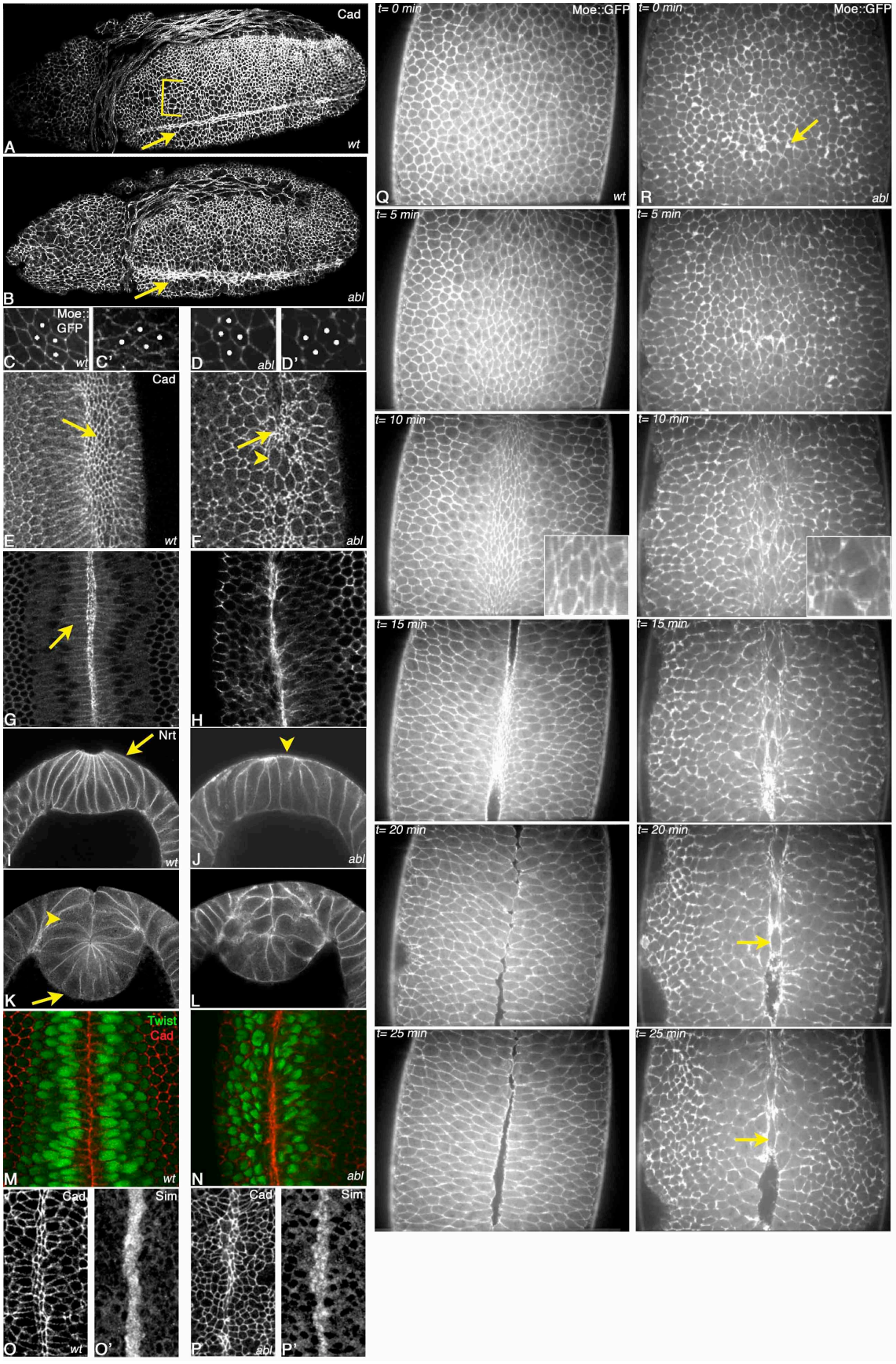
Sweeton, D., Parks, S., Costa, M. and Wieschaus, E. (1991). Gastrulation in Drosophila: the formation of the ventral furrow and posterior midgut invaginations. *Development* **112**, 775-89.

Wang, Y., Miller, A. L., Mooseker, M. S. and Koleske, A. J. (2001). The Abl-related gene (Arg) nonreceptor tyrosine kinase uses two F-actin-binding domains to bundle F-actin. *Proc Natl Acad Sci U S A* **98**, 14865-70.

Woodring, P. J., Hunter, T. and Wang, J. Y. (2003). Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. *J Cell Sci* **116**, 2613-26.

Zallen, J.A., and Wieschaus, E. (2004). Patterned gene expression directs bipolar planar polarity in *Drosophila. Dev. Cell* **6.** 343-55.

Fig.4.1. Abl is required for apical constriction in the ventral furrow. (A,B) Low magnification, germband extended embryos, anterior to left, dorsal up, DE-Cad. (A) Wild-type, bracket- germband, arrow- ventral midline. (B) *abl* mutant. Arrow shows irregular midline (compare with A). (C-D') High magnification of live embryos, germband (region near bracket in A), anterior to left, dorsal up, Moesin::GFP . (C-C') Wild-type, prior to (C) and after (C') cell intercalation. Note the rearrangement of cell contacts in the quartet of cells marked with white dots. (D-D') *abl* mutant, prior to (D) and after (D') cell intercalation. Similar junctional rearrangements occur as in wild-type (compare C vs. D, C' vs. D'). (E,F,G,H) Grazing sections, ventral, DE-Cad. (E,F) Apical, early gastrulation. (E) Wild-type, arrow shows apically constricting mesodermal cells, all uniformly changing shape. (F) *abl* mutant, arrow- constricting cells, arrowhead- un-constricted cells in the region of the mesoderm, identifying a ventral furrow defect (compare E vs. F). (G, H) Sub-apical, mid-gastrulation. (G) Wild-type, lateral mesodermal cells (arrow) orient parallel to the furrow. (H) *abl* mutant, lateral mesodermal cells are drawn towards the furrow at varying angles. (I, J, K, L) Cross-sections, ventral, Neurotactin (Nrt). (I,J) Early gastrulation. (I) Wild-type. Central mesodermal cells constrict and point towards the furrow. Arrow-outermost constricting cells exhibit dramatic shape changes. (J) *abl* mutant. Arrowhead-non-constricted cell. (K,L) Late gastrulation. (K) Wild-type, arrow- wedge shaped constricted cells in center of furrow, arrowhead- unconstricted lateral furrow cells, aligned perpendicular to furrow. (L) *abl* mutant. Asymmetric cell shapes are seen throughout the furrow. (M,N) Twist (Green) expression is similar between wild-type (M) and *abl* mutant (N) mesoderm (Red, DE-Cad). (O-P') Midline morphogenesis (seen with DE-Cad, O,P) and joining (seen with Single-minded, Sim) in wild-type (O-O') and *abl* mutants (P-P'). *abl* mutants occasionally exhibit irregular cell shapes in the midline (O vs. P), yet the two rows midline cells properly join (O' vs. P'). (Q, R) Stills from live imaging of wild-type (Q) and *abl* mutants (R) expressing Moesin::GFP. Comparison of insets at t=10 minutes (Q vs. R) highlights the asymmetry in cell shape change in *abl* mutants. *abl* mutants also exhibit ectopic F-actin patches at the onset of furrow formation (R, t=0 min.), and persistent mesoderm (R, t=20, 25 min., arrows) in the furrow.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t= 10 min
t= 10 min
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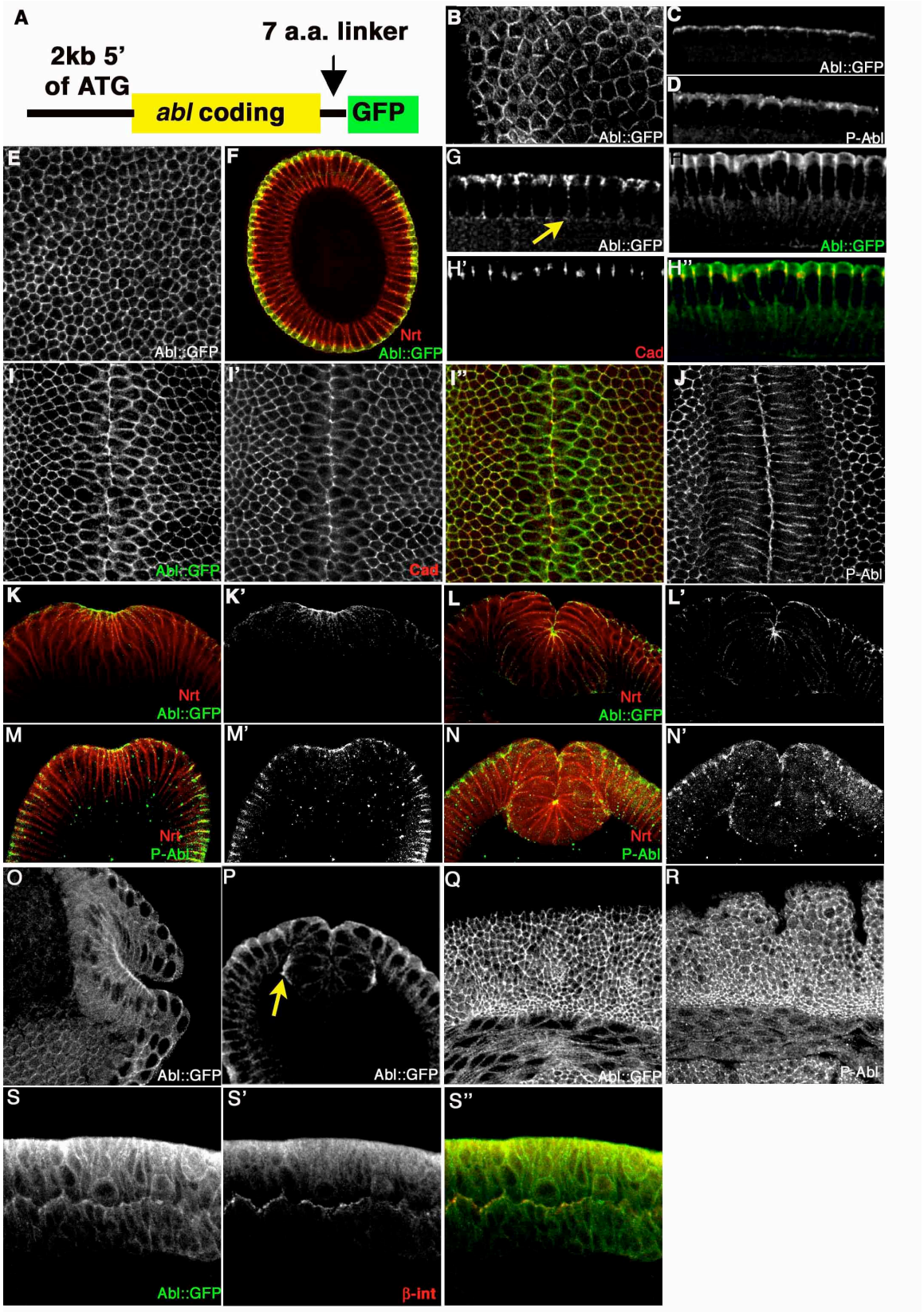
t= 25 min

Fig. 4.2. Abl localization and activity concentrates apically in embryonic epithelia. (A) Schematic of *abl::GFP* construct. 2kb of 5'UTR acts as an endogenous promoter, and a short 7 amino acid (a.a.) linker bridges *abl* and *egfp* coding sequences. (B-D) Syncytial blastoderm. (B) Grazing section, Abl::GFP at the cell cortex. (C,D) Cross-sections. Abl::GFP (C) and Phospho-Abl (P-Abl, D) localize apically. (E-H') Cellularization. (E) Grazing section, Abl::GFP at the cell cortex. (F-H') Cross-sections. (F) Low magnification, Abl::GFP (green) localizes apically throughout the embryo (Red, Nrt marks all membrane). (G-H'') High magnification (G) At mid-cellularization, Abl::GFP can be seen apically but also faintly in basal furrow canals (arrow). (H-H'') Abl::GFP (H, H'', green) localize apically and partially overlap with adherens junctions (H', red in H'', DE-Cad). (I-N') Ventral furrow. (I-J) Grazing sections. Abl::GFP (I, green in I'') colocalizes with constricting adherens junctions (DE-Cad, I', red in I''). (J) P-Abl accumulates strongly in the furrow. (K-N') Cross-sections. Red- Nrt. (K-L') Abl::GFP (green in K, L) accumulates at sites of apical constriction early (K-K') and late (L-L') in ventral furrow formation. (M-N') P-Abl (green in M, N) accumulates at sites of apical constriction early (M, M') and late (N, N') in ventral furrow formation. (O) Abl::GFP localizes to apically constricting posterior midgut cells. (P) Late in ventral furrow formation, Abl::GFP also resides at a basal structure (arrow). (Q-R) Germband extended embryos, apical grazing sections. Abl::GFP (Q) and P-Abl (R) localize prominently to the cell cortex. (S-S'') Abl::GFP (S, green in S') overlaps with Beta-integrin (S', red in S'') basally in epithelial cells at gastrulation. All Abl::GFP visualization is with anti-GFP.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of ATG
7 a.a. linker
abl coding
GFP
B
Abl::GFP
C
Abl::GFP
D
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F
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Abl::GFP
G
Abl::GFP
H
Abl::GFP
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R
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Abl::GFP
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S''

Fig. 4.3. Myosin and RhoGEF2 fail to uniformly assemble a contractile network in *abl* mutants. (A-F'') Cross-sections. (A-D) Low magnification. (A-B) Late cellularization. Myosin localizes basally in both wild-type (A) and *abl* mutants (B). (C-D) Early ventral furrow formation. Myosin begins to accumulate apically in both wild-type (C) and *abl* mutants (D). (E-F'') Close-ups, ventral furrow. (E-E'') Wild-type. Myosin (E, green in E'') accumulates apically in cells that constrict (seen with Nrt- E', red in E''). (F-F'') *abl* mutant. Myosin (F, green in F'') accumulates strongly in constricting cells arrow in (F), but only faintly in non-constricting cells (arrowhead in F). Nrt- F', red in F''. (G-H'') Grazing sections. (G-G'') Wild-type. Myosin (G, green in G'') evenly coats the apical surface (seen with Armadillo- G', red in G'') of all constricting mesoderm. (H-H'') *abl* mutant. Myosin (H, green in H'') concentrates most at sites of apical constriction (seen with arrow and with Armadillo- H', red in G'') but is only faintly present elsewhere (arrowhead). (I-N'') Cross-sections. (I-L) Low magnification. RhoGEF2 (GEF)- green, Nrt- red. (I-K) Wild-type. GEF localizes basally at the end of cellularization but leaves the basal surface of mesoderm (I), then localizes diffusely and apically everywhere (J), before accumulating strongly at the apical surface of soon to constrict mesoderm (K). (L) GEF also accumulates apically on the ventral surface of *abl* mutants prior to furrow formation. (M-N'') High magnification. (M-M'') Wild-type. GEF (M, green in M'') localizes strongly to all apically constricting cells (seen with Nrt- M', red in M''). (N-N'') *abl* mutant. GEF (N, green in N'') localizes strongly only in constricting cells (arrow in N), but is more diffuse in non-constricting cells (arrowhead in N). Nrt- N', red in N''. (O-P) Grazing sections, ventral. (O) Wild-type. Apical GEF localization is uniform throughout the furrow. (P) *abl* mutant. Apical GEF is strong in places (arrow), and weaker in others (arrowhead). (Q,R) Cross sections, ventral, Phospho-Myosin (P-Myo). Constant level imaging reveals similar levels of active myosin at sites of apical constriction in wild-type (Q) vs. *abl* mutants (R).

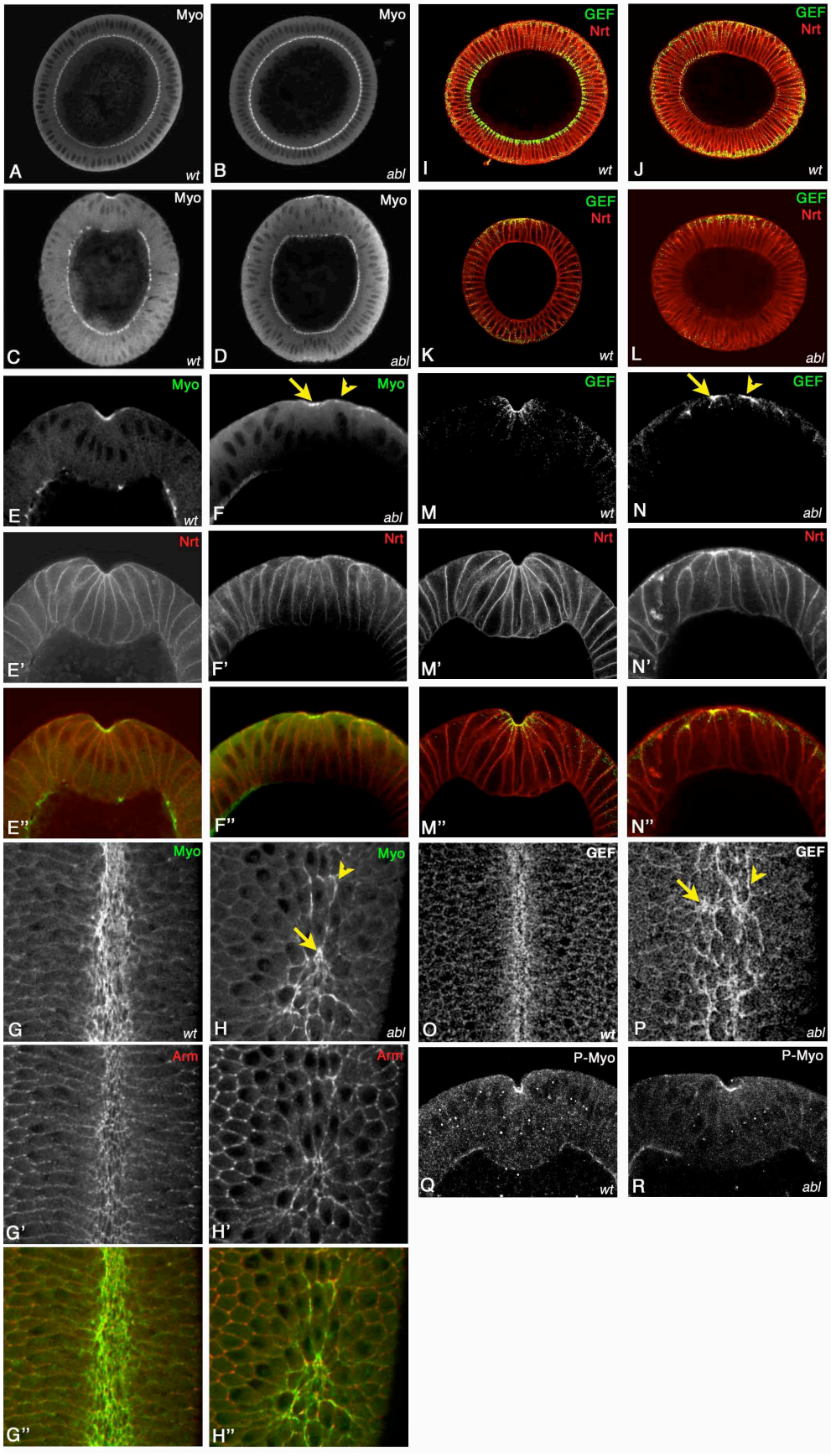
Myo
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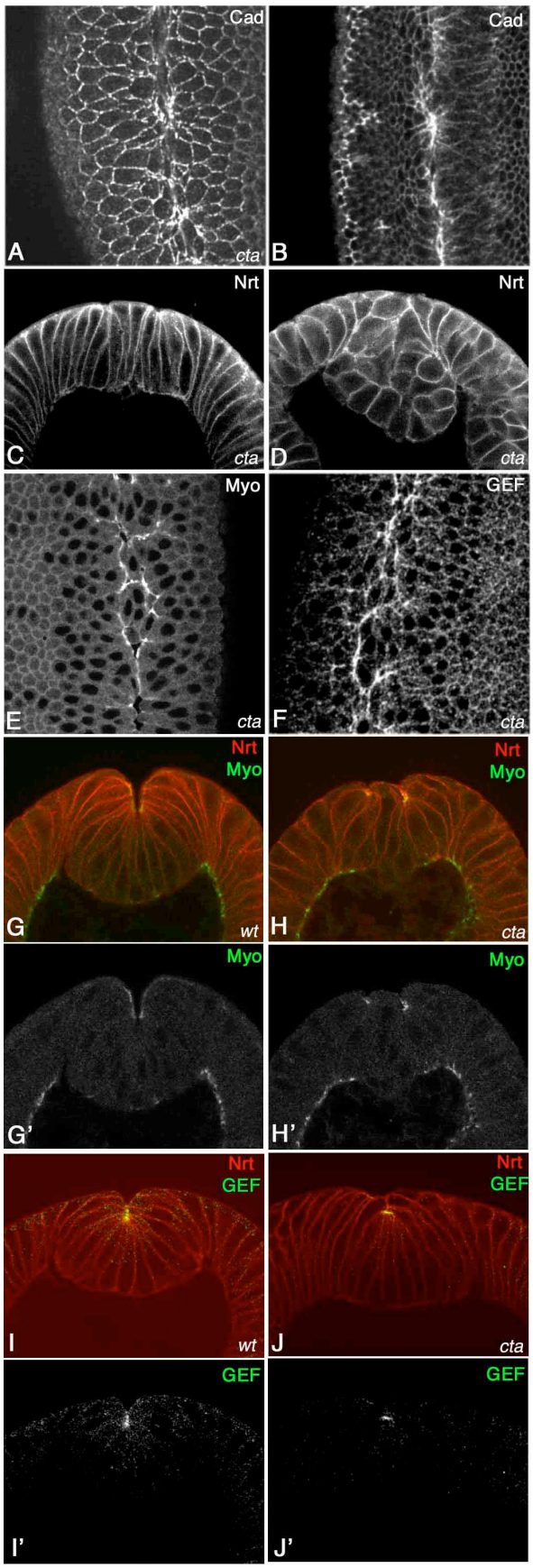
Fig. 4.4. *cta* mutants resemble *abl* mutants. (A, B) Grazing sections, DE-Cad. (A) Early gastrulation, apical. *cta* mutants exhibit large, unconstricted cells (compare to *abl*, Fig. 1F). (B) Late gastrtulation, sub-apical. *cta* mutants internalize mesoderm at varying angles (compare to *abl,* Fig. 1H). (C, D) Cross-sections, Nrt. (C) Early gastrulation. *cta* mutants exhibit un-constricted cells in between constricting cells (compare with *abl,* Fig. 1J). (D) Late gastrulation. *cta* mutants exhibit asymmetry in mesodermal cell shape (compare with *abl,* Fig. 1L). (E, F) Grazing sections. (E) Apical Myosin and (F) GEF accumulation in the ventral furrow is disrupted (compare to *abl*- E vs. Fig. 3H, F vs. Fig. 3P). (G-J') Cross-sections. (G-H') Myosin (G', H', green in G, H) levels are comparable at sites of apical constriction (cell shape seen with Nrt- red in G, H) between wild-type (G-G') vs. *cta* mutants (H-H'), but in *cta* mutants Myosin is uneven, similar to *abl* (see Fig. 3F). (I-J') GEF (I', J', green in I, J) levels are comparable at sites of apical constriction (see Nrt- red in I, J) between wild-type (I-I') vs. *cta* mutants (J-J'), but in *cta* mutants GEF is uneven, similar to *abl* (see Fig. 3N).


Cad
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GEF
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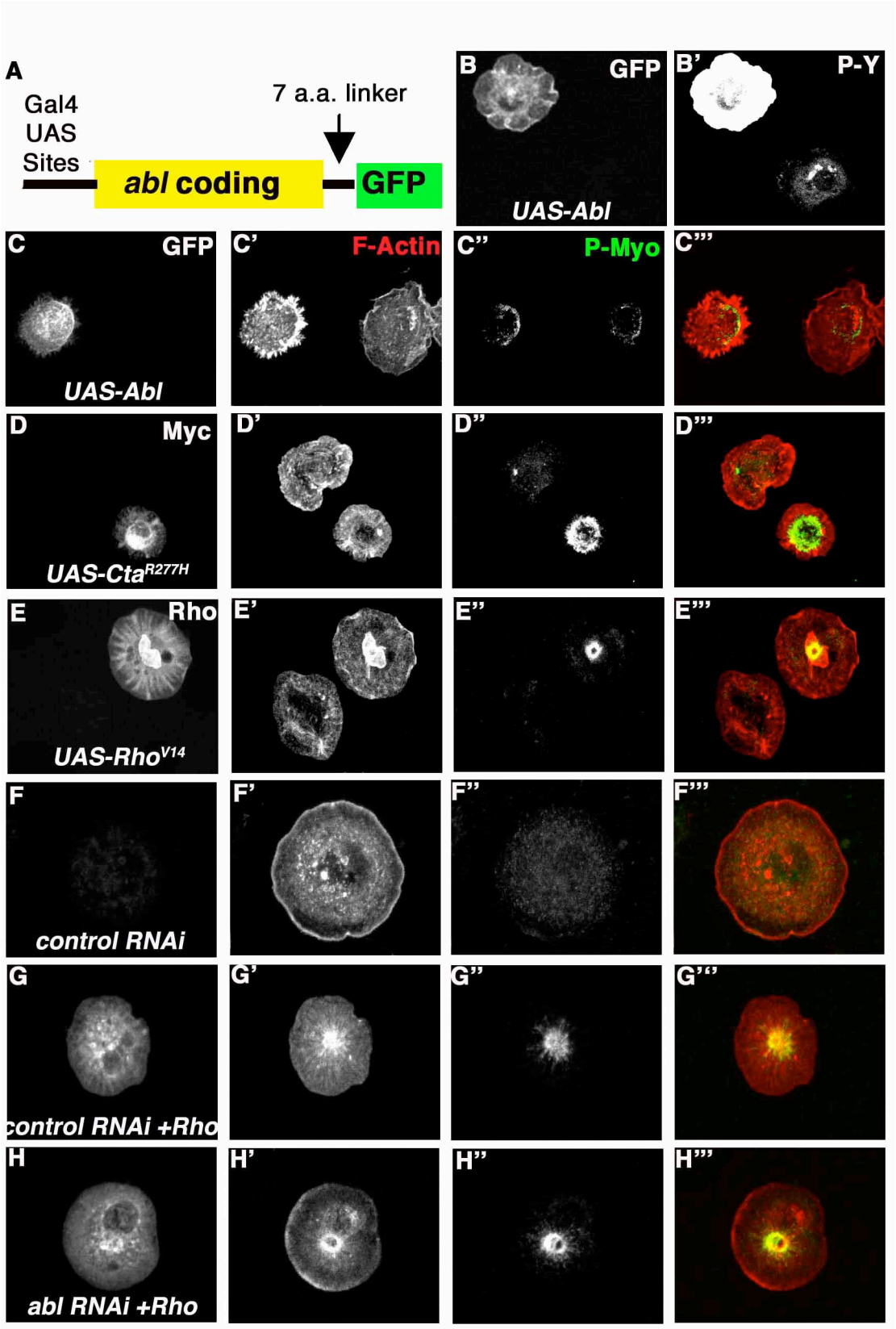
Fig. 4.5. In S2 cells, Abl promotes actin accumulation while Cta promotes myosin accumulation. (A) Scheme for UAS-Abl::GFP. The same fusion diagramed in Fig. 2A, but the endogenous promoter is replaced with Gal4 UAS sites. (B-B') UAS-Abl cells (GFP positive, B) display increased Abl activity, as assayed by Phospho-tyrosine (P-Y, B'). (C-E''') Actin (C', D', E') and P-Myo (C'', D'', E'') in UAS-Abl (C-C'''), UAS-Cta (D-D'''), and UAS-Rho (E-E''') transfected cells. (C''', D''', E''') Merged images showing actin in red and P-Myo in green. Abl-transfected cells (GFP, C) display increased levels of actin (C', C'''), but not P-Myo (C'', C'''). Cta-transfected cells (Myc, D) display increased levels of P-Myo (D'', D''') but not actin (D', D'''). Rho-transfected cells (Rho, E) exhibit both increased actin (E', E''') and P-Myo (E'', E'''). However, both the increased actin and P-Myo in Rho-transfected cells tightly organize into a central ring-like structure (compare E' vs. C', E'' vs. D''). (F-H''') *abl* RNAi does not disrupt the Rho gain-of-function phenotype. (F-F''') Control RNAi, showing low levels of Rho (F), peripheral actin (F', red in F'''), and diffuse P-Myo (F'', green in F'''). (G-G''') Control RNAi plus active Rho, showing increased Rho (G vs. F), and concentrated actin (G', red in G''') and P-Myo (G'', green in G'''). (H-H''') *abl* RNAi plus active Rho. Rho (H), Actin (H', red in H''') , and P-Myo (H'', green in H''') are identical to G-G'''.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abl coding
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B
GFP
UAS-Abl
B'
P-Y
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UAS-Abl
C'
F-Actin
C''
P-Myo
C'''
D
Myc
UAS-Cta^R277H
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H''
H'''

Fig. 4.6. Actin accumulates ectopically in *abl* and *RhoGEF2* mutants, but not in *cta* mutants. Wild-type (A-D), *abl* mutants (E-H), *cta* mutants (I-L), and *RhoGEF2* mutants. (A-A'', E-E'', I-I'', M-M'') Late cellularization. Actin (A, E, I, M, red in A'', E'', I'', M'') localize basally in all genotypes, but accumulates ectopically in *abl* (E) and *RhoGEF2* (M) mutants, not in wild-type (A) or *cta* mutants (I). Ena (A', E', I', M', green in A'', E'', I'', M'') exhibits diffuse localization in wild-type (E'), *cta* (I'), and *RhoGEF2* mutants (M'), but accumulates apically in *abl* mutants (E'). (B, F, J, N) Actin, ventral furrow stage, low magnification. Ectopic apical actin is seen in *abl* (F) and *RhoGEF2* (N) mutants, but not in wild-type (B) or *cta* (J) mutants. (C, G, K, O) Ena, ventral furrow, high magnification. (C) Ena localizes apically in non-mesodermal cells (arrow), but remains diffuse in mesodermal cells (arrowhead). (G) *abl* mutants accumulate Ena in the mesoderm. (K, O) Neither *cta* mutants (K) nor *RhoGEF2* mutants (O) accumulate ectopic mesodermal Ena. (D, H, L, P) Actin, ventral furrow, high magnification. (D) Actin is highly polarized in wild-type, and disappears from its previous basal location (arrow, compare with A). (H, P) Actin accumulates apically in *abl* mutant (H) and *RhoGEF2* mutant (P) ventral furrows. (L) *cta* mutants do not display an excess apical actin phenotype in the ventral furrow. (Q-Q') *RhoGEF2* mutants, late gastrulation, low magnification. Excess apical actin (Q, red in Q'') colocalizes with Ena (Q', green in Q'') in ectopic structures. (R) Heterozygosity of *ena* suppresses the *abl* ventral furrow phenotype (DE-Cad).

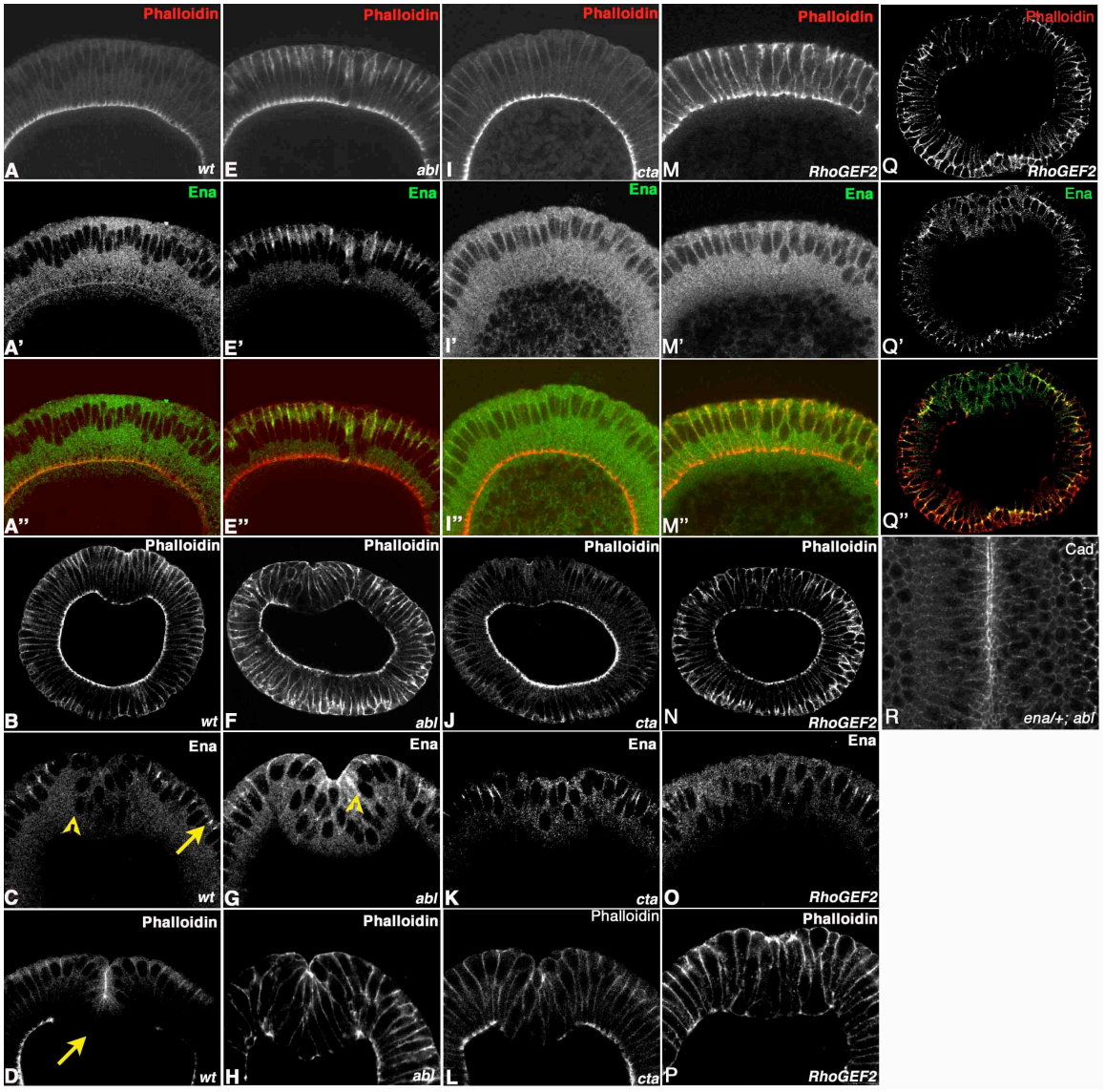
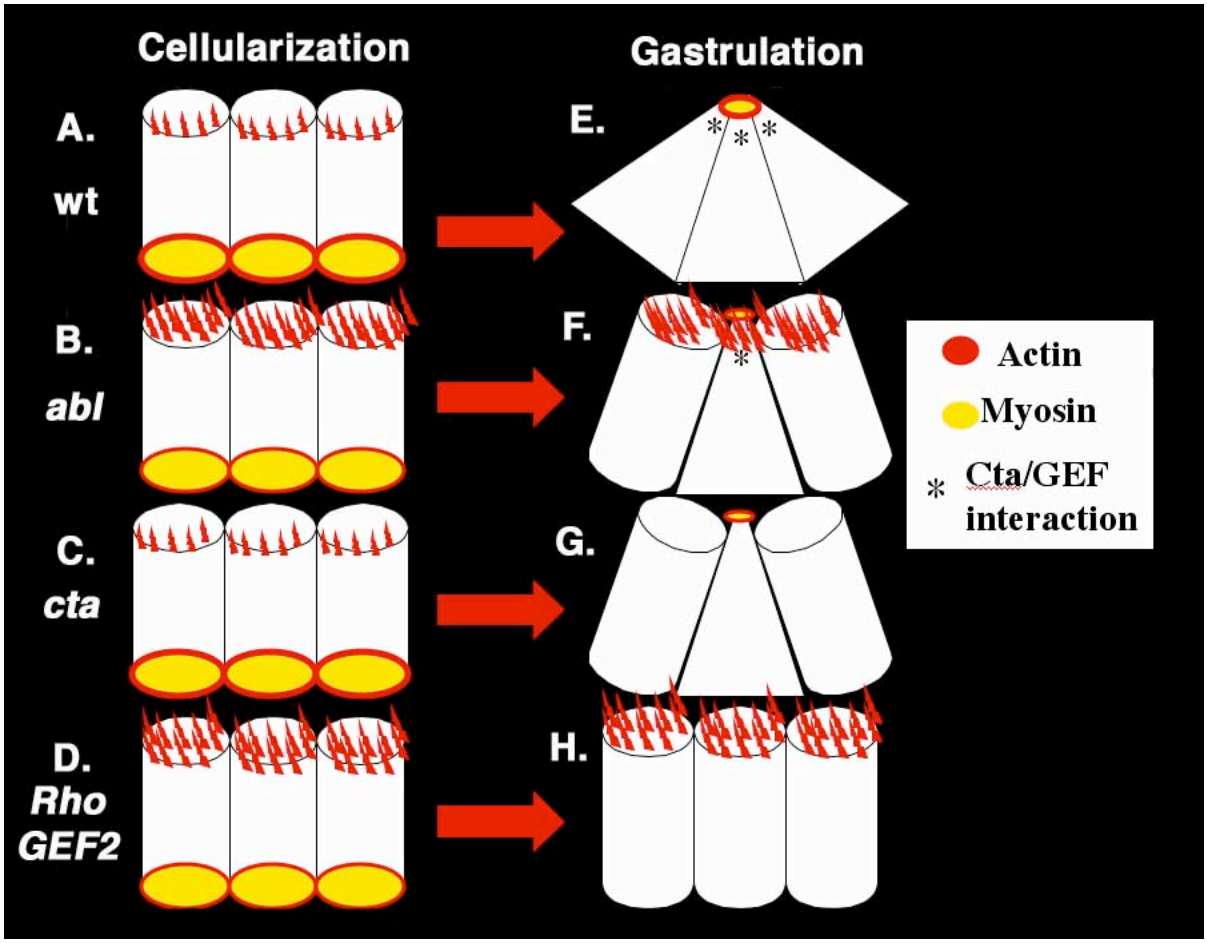

Phalloidin
A wt
E abl
I cta
M RhoGEF2
Q RhoGEF2
Ena
A'
E'
I'
M'
Q'
A''
E''
I''
M''
Q''
B wt
F abl
J cta
N RhoGEF2
Cad
R ena/+; abl
C wt
G abl
K cta
O RhoGEF2
D wt
H abl
L cta
P RhoGEF2

Fig. 4.7. A meachanistic model of ventral furrow formation. Cellularization (A-D) and gastrulation (E-H) in the mesoderm in wild-type (A,E), *abl* mutants (B,F), *cta* mutants (C, G), and *RhoGEF2* mutants (D, H). A. During cellularization, actin (red) and myosin (yellow) concentrate in a ring-like pattern in the basal furrow canal, while a small amount of actin resides in apical microvilli. B. Excess apical actin forms in *abl* mutants during cellularization. C. Actin and myosin are normal in *cta* mutants at cellularization. D. *RhoGEF2* mutants exhibit *abl*-like actin defects at cellularization. E. In mesodermal cells, actin and myosin translocate apically, and are stabilized by Cta/RhoGEF2 interactions (star). F. In an *abl* mutant, Cta/GEF interactions are transiently blocked by apical actin, resulting in some non-constricted cells. G. In *cta* mutants, lack of Cta/GEF interactions results in only transient apical myosin contraction, resulting in some non-constricted cells. H. In *RhoGEF2* mutants, the combination of no Cta/GEF interactions and the build-up of apical actin synergize to completely block apical constriction.


Cellularization
Gastrulation
A.
wt
B.
abl
C.
cta
D.
Rho
GEF2
E.
F.
G.
H.
Actin
Myosin
Cta/GEF
interaction

CHAPTER 5

CHARACTERIZATION OF ABL LOCALIZATION AND ACTIVATION

Preface

The following describes unpublished work in which I surveyed Abl localization and activation in embryos after gastrulation (a pre-gastrulation characterization is provided in chapter 4). To do so, I utilized two tools: Abl::GFP (described in detail in chapter 4) and a phospho-specific mammalian Abl antibody, for which I provide a detailed characterization here. Further, I utilized the phospho-Abl antibody to initiate studies of candidate upstream Abl activators. The characterization of the Phospho-Abl antibody is included in a manuscript that is currently in preparation. The remainder of this work will be continued by Ed Rogers, a post-doc in the lab.

Introduction

Abl family kinases have been functionally linked to the actin cytoskeleton in many contexts. Studies in cultured mammalian cells have revealed Abl family proteins in different actin-based structures, such as filopodia and membrane ruffles (reviewed in Woodring et al., 2003). These observations suggest that Abl may preferentially localize to sites of actin reorganization during morphogenesis. Importantly, however, examining the localization of all Abl kinase in a cell alone does not necessarily reveal where this kinase is active. Crystal

structure studies of Abl bear this out, as Abl appears to require several activation events to convert to the active form (reviewed in Harrison, 2003). Thus, it is possible that a majority of Abl protein is in fact not active. Therefore, comparing the localization of active and total Abl may lend additional detail to studies of Abl function. Further, studying Abl activation also raises the question of how Abl is activated. Studies in cultured mammalian cells suggest that the receptor tyrosine kinases EGFR and PDGFR as well as the non-receptor tyrosine kinase Src may activate Abl (Tanis et al 2003; Plattner et al 1999). However, it is not clear what signals activate Abl in the context of an intact animal.

In the work described below, I initiated a survey of Abl localization and activation in *Drosophila* embryos after gastrulation. I identified a striking localization pattern of Abl between daughter cells in late cytokinesis. Abl's localization at this site differs from other late cytokinesis markers, suggesting that this late mitotic structure is complex in nature. Further, I demonstrated that a phospho-specific mammalian Abl antibody recognizes active fly Abl. Active Abl localizes to the apical cell cortex and central nervous system in post-gastrulating embryos. I then used this active Abl antibody to show that overexpression of either *Drosophila Src42* or *pvr* can stimulate Abl activation. Taken together, these results show that Abl is active in the apical cell cortex, identify a possible role for Abl as a regulator of cytokinesis, and provide the first evidence of upstream activators of Abl in an intact animal.

Materials and methods

Immunofluorescence

Embryos were fixed in 1:1 PBS+3.7% formaldehyde:heptane for 20 min. Embryos were methanol-devitellinized, blocked and stained in PBS/1% goat serum/0.1%Triton-X-100. Antibodies: anti-phospho (Y412) (1:250), anti-Rho (1:250), anti-phosphotyrosine (1:1000), anti-phosphohistone H3 (1:500), anti-anillin (1:100), anti-acetylated tubulin (1:2000), and anti-GFP (1:2000), anti-engrailed (1:50).

Immunobloting

Embryo extracts were separated on 6% SDS-PAGE, transferred to nitrocellulose, and probed with anti-Abl (1:100), anti-Phospho(Y412) Abl (1:2500), or anti-Pnut (1:30).

Results

Abl localizes to distinct structures late in mitosis

Our previous analysis of Abl localization centered on the blastula and gastrula stages of development (Chapter 4). I extended this analysis to later stages of development. As during earlier stages, I found Abl::GFP to localize to the apical cell cortex (Fig. 1A, Fig. 4A, B). Unlike during gastrulation, Abl::GFP is no longer uniformly around the cortex in all epithelial cells but is seen in puncta at "tri-cellular junctions" where several cells meet (Fig. 4B, arrow).

One unexpected result was the identification of a striking pattern of Abl localization in dividing cells. At the end of cellularization cells arrest in G2 of the cell cycle. After gastrulation, embryonic cells enter mitosis in a stereotyped pattern, with groups of cells in "mitotic domains" entering mitosis synchronously (Foe, 1989). At the apical cortex, I found Abl to brightly decorate the boundary between two daughter cells (Fig. 1A, arrow) during late telophase. I confirmed this observation by co-staining with phospho-histone H3, which

marks early mitotic cells. This marker clearly marks large, pre-anaphase cells (Fig. 1A), but is absent in recently divided daughter cells within a mitotic domain. Cells lacking phospho-histone staining contain a bright boundary of Abl::GFP localization.

My first hypothesis was that Abl may localize to the contractile ring during cytokinesis. To test this hypothesis, I co-localized Abl::GFP with the contractile ring marker Rho1 (Fig. 1B-E). This analysis revealed that Abl:GFP does not reside in contractile rings when they are first formed (Fig. 1B, compare Rho1 vs. Abl::GFP). However, shortly after contractile ring formation, Abl::GFP does overlap with Rho1 (Fig. 1C,D) and the contractile ring marker Anillin (Fig. 1E), residing between daughter cells. The contractile ring is also bisected by acetylated tubulin (Fig. 1E, F), which marks the spindle midzone, a remnant of the mitotic spindle and marker between recently divided cells.

Abl::GFP localization within these late mitotic structures differs from Rho1 and anillin. While these contractile ring components progressively re-localize into a dot like structure very late in telophase (Fig. 1D,E for Rho1 and Fig. 1F for Anillin), Abl::GFP localization occupies a broader domain, possibly flanking this dot-like structure (Fig. 1E, compare Abl::GFP and Rho1). Thus, Abl appears to localize between daughter cells in a temporally and spatially distinct manner to known contractile ring proteins. This localization may specifically mark the newly assembled membrane separating the two daughters. However, the apical membrane protein DE-Cad is not as enriched at the same location as Abl (Fig. 1B-D, compare Abl::GFP vs. DE-Cad).

Phospho(Y412) Abl: visualizing active Abl in fly embryos

As a second approach to visualizing Abl in late embryos, I utilized a tool that allowed us to visualize active Abl. I obtained a commercial antibody against phosphorylated Y412

in human c-Abl, a residue that contributes to mammalian Abl activation (reviewed in Woodring et al., 2003). Alignment of the peptide used to produce this mammalian antibody with *Drosophila* Abl shows that 8/10 residues are identical, with a tyrosine in the position equivalent to Y412 (Fig. 2A). To demonstrate that this antibody recognizes fly Abl, we performed Western blot analysis from wild-type and Abl over-expressing embryonic extracts. As for anti-Abl, phospho(Y412) Abl recognizes a doublet at ~180 kDa in embryos overexpressing wild-type Abl and in wild-type embryos (Fig. 2B, star, lanes 1 and 4 from left). It also cross-reacts with other proteins above 180 kDa (Fig. 2B, arrows).

Further, this antibody specifically recognizes active Abl. This is revealed by the increase in signal in embryos expressing a hybrid mammalian-fly fusion of the overactive BCR-Abl translocation (Fogerty et al 1999, Fig. 2B lane 2, our generic fly Abl antibody does not recognize BCR-Abl). Additionally, while embryos expressing kinase dead Abl contain elevated levels of total Abl (Fig. 2B lane 3), levels of phospho-Abl are unaltered (Fig. 2B, compare lanes 1 vs. 3 for phospho-Abl). Finally, when Abl activation is triggered by overexpression of wild-type Abl or mis-expression of the p185 or p210 isoforms of BCR-Abl (which are constitutively active), active Abl is enriched at the cortex of expressing cells (Fig. 2C, D, E, arrows). Thus, an antibody directed against active mammalian Abl also recognizes active fly Abl.

Having confirmed that the P(Y412)Abl antibody recognizes active Abl, we next examined the sub-cellular localization of active Abl in embryos. When Abl activation is forced, by overexpression of either wild-type or the p185 or p210 isoforms of BCR-Abl, active Abl is enriched at the cortex of expressing cells (Fig. 2C,D, E, arrows). The same localization trend is true in wild-type embryos throughout embryonic development. Active

Abl localizes to the cell cortex during gastrulation (Fig. 3A), germband extension (Fig. 3C), as well as during (Fig. 3E) and after (Fig. 3G) dorsal closure.

Active Abl is also seen at specific sub-cellular locations in embryos. Co-localization with Abl::GFP confirms that the Abl::GFP that accumulates between daughter cells is active (Fig. 4B, arrowheads). Active Abl is also seen in puncta at tricellular junctions (Fig. 3B, D), much like total Abl (Fig. 4B, arrowheads). This localization pattern overlaps that of the Abl target Ena (Fig. 4C, arrows). One place where active Abl and Ena localization differ occurs during dorsal closure. While Ena accumulates in puncta at the leading edge (Fig. 4D, arrow) and is enriched in "segmental groove" cells (Fig. 4D, arrowhead), Abl activation is not similarly enriched at these locations (Fig. 4D). Finally, active Abl also localizes to the central nervous system axons (Fig. 3H), as does bulk Abl protein (Bennett and Hoffmann, 1992). In summary, use of a mammalian phospho-specific Abl antibody recognizes active Abl protein. Abl is active at the cell cortex and in CNS axons, and overlaps in many places with the Abl target Ena.

Src42 and PVR are candidate activators of *Drosophila* Abl

The ability to visualize active Abl presents the opportunity to identify upstream signals that activate Abl. Previous work in cultured cells identified receptor tyrosine kinase signaling by epidermal growth factor (EGF) and platelet derived growth factor (PDGF) as upstream signals for Abl activation (Plattner et al., 1999). To test whether signaling from these growth factors could activate Abl in *Drosophila*, I expressed constitutive active forms of the receptors for both growth factors (EGFR and PVR) in the embryonic epidermis. To internally control for expressing vs. non-expressing cells, I used the engrailed-GAL4 driver,

which drives expression of each construct in alternating stripes of cells (Fig. 5, red channel in all merged images).

Expression of PVR resulted in an increase in PAbl signal in engrailed-positive cells (Fig. 5A, A'). In contrast, expression of active EGFR with the same driver did not change PAbl signal in engrailed-positive cells (Fig. 5C, C'). However, I can not rule out EGFR as an Abl activator, as expression of this tyrosine kinase, unlike PVR expression, did not elevate phospho-tyrosine levels (Fig. 5B vs. D), calling into question how effectively the receptor was activated. I also tested several other EGFR overexpression constructs, including tagged constructs to verify expression, and none showed elevated phospho-tyrosine. Thus, while EGFR expression in our hands does not elevate phospho-tyrosine or phospho-Abl levels, overexpression of PVR results in increased active Abl.

Next, we examined potential downstream effectors of receptor tyrosine kinases for potential Abl activation. The non-receptor tyrosine kinase Src has been shown to activate c-Abl (Tanis et al., 2003), and PDGF activation of Abl appears to be dependent on Src (Plattner et al., 2004). Thus, PDGF may signal through Src to activate Abl. To test if Src behaves as an Abl activator in *Drosophila,* we mis-expressed constitutive active Src42 in engrailed stripes. As for PVR overexpression, phospho-tyrosine levels (Fig. 5F) and Abl activation increase in response to Src overexpression (Fig. 5E, E').

The MAP kinase pathway is a well-known downstream target of EGF signaling (reviewed in Schlessinger, 2000). While we did not see activation of Abl by EGFR overexpression, we further tested whether EGF-dependent signaling could activate Abl. To test this, we overexpressed wild-type Raf kinase. As for EGFR mis-expression, Raf

overexpression does not alter levels of Abl activation (Fig. 5G). Thus, while PVR and Src are sufficient to activate Abl, EGFR and Raf signaling do not activate Abl.

Discussion

The above experiments represent initial studies of Abl localization and activation in the post-gastrulating embryo. Many significant Abl-dependent processes occur during these stages, such as germband retraction and dorsal closure in the epidermis and axon guidance in the central nervous system. By observing when and where Abl is present and active, we better understand mutant phenotypes and interpret the role of Abl in developmental processes.

A role for Abl in cytokinesis?

Our previous work showed a clear role for Abl during the cytokinesis-like events of syncytial mitosis and cellularization. While these events differ from standard cytokinesis, they do involve reorganization of the actin cytoskeleton to separate nuclei. In Abl's absence, actin fails to assemble into membrane furrows, resulting in transient multinucleated cells (Grevengoed et al., 2003). Whether Abl plays a similar role in late embryogenesis remains to be determined. While some late stage *abl* mutant embryos contain multinucleated cells, it is unclear whether these are remnants of blastoderm stage defects. A careful analysis will be needed to determine whether Abl's localization to cleavage furrow remnants represents a role in cytokinesis.

The late localization of Abl between daughter cells suggests that if Abl participates in cytokinesis, it does so at the very terminal step. Among the class of late-cytokinesis regulators are Anillin and Rho (Echard et al., 2004), which overlap Abl localization between

daughter cells. These cytokinesis regulators were identified in an RNAi screen in S2 cells, which did not identify Abl as having this phenotype (Echard et al., 2004). However, several knockdowns in this screen produced subtle phenotypes. It is intriguing to think of how cytokinesis would proceed if a similar knockdown were performed in conjunction with Abl. One possible role for Abl might be regulation of membrane deposition of the reformation of adherens junctions between the daughter cells.

Abl activation: an opportunity for new insights

The ability to analyze active Abl will greatly enhance our understanding of Abl function. By comparing both total and active Abl, we may better understand where and when Abl is active. If specific localization patterns of Abl activity are observed in the future, they may be predictive of Abl-dependent processes. The lack of Abl enrichment with Ena in leading edge puncta and in segmental groove structures is interesting given our lab's findings that Abl is required for both leading edge and segmental groove morphogenesis (Grevengoed et al 2001; Stevens et al, in preparation). Perhaps a scaffold for Ena is enriched in these cells, and Abl is still playing its role as a negative regulator at these locations as in other places.

As preliminary data suggests, the active antibody may serve as a helpful tool for identifying Abl activators. The *Drosophila* community has created a great resource known as an "EP" library, which is a set of stocks for overexpression of many genes in the genome. Such a library could be screened through with the Phospho-Abl antibody to identify potential Abl activators; given the scale of the effort this might be done best by targeting candidate genes.

A potential cytoskeletal signaling pathway involving Abl

Our preliminary results implicate Src and Pvr as activators of Abl. This result must now be tested more rigorously to functionally connect these kinases to Abl activation. For instance, mis-expression of these candidate activators should affect Ena localization. Further, the mutant phenotypes of candidate activators should resemble, in part, *abl* phenotypes. A recent study of *src42* loss of function is encouraging, as these mutant embryos display germband retraction defects (Takahashi et al., 2005), thus resembling *abl* mutants (Grevengoed et al 2001).

An interesting mechanistic picture of PVR function is emerging in another fly model of morphogenesis. In migrating border cells of the ovary, both EGFR and PVR have been shown to act in a non-canonical (not through MAP kinase) signaling pathway to reorganize the cytoskeleton (reviewed in Gupta and Schupbach, 2001). Interestingly, both a Src family member (Somogyi and Rorth, 2004) and Abl (Rorth et al., 1998) have also been implicated in border cell migration. Our results suggest that Abl activation is downstream of both PVR and Src, and is MAPK-independent. This raises the possibility that Abl may lie downstream of a receptor tyrosine kinase pathway of cytoskeletal regulation involving at least PVR and Src.

References

Bennett, R. L. and Hoffmann, F. M. (1992). Increased levels of the Drosophila Abelson tyrosine kinase in nerves and muscles: subcellular localization and mutant phenotypes imply a role in cell-cell interactions. *Development* **116**, 953-66.

Echard, A., Hickson, G. R., Foley, E. and O'Farrell, P. H. (2004). Terminal cytokinesis events uncovered after an RNAi screen. *Curr Biol* **14,** 1685-93.

Foe, V. (1989). Mitotic domains reveal early commitment of cells in Drosophila embryos. *Development* **107,** 1-22.

Fogerty, F. J., Juang, J. L., Petersen, J., Clark, M. J., Hoffmann, F. M. and Mosher, D. F. (1999). Dominant effects of the bcr-abl oncogene on Drosophila morphogenesis. *Oncogene* **18,** 219-32.

Grevengoed, E. E., Fox, D. T., Gates, J. and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. *J Cell Biol* **163,** 1267-79.

Grevengoed, E. E., Loureiro, J. J., Jesse, T. L. and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in Drosophila. *J Cell Biol* **155,** 1185-98.

Gupta, T. and Schupbach, T. (2001). Two signals are better than one: border cell migration in Drosophila. *Dev Cell* **1,** 443-5.

Harrison, S. C. (2003). Variation on an Src-like theme. *Cell* **112,** 737-40.

Plattner, R., Kadlec, L., DeMali, K. A., Kazlauskas, A. and Pendergast, A. M. (1999). c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. *Genes Dev* **13,** 2400-11.

Plattner, R., Koleske, A. J., Kazlauskas, A. and Pendergast, A. M. (2004). Bidirectional signaling links the Abelson kinases to the platelet-derived growth factor receptor. *Mol Cell Biol* **24,** 2573-83.

Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G. M., Weigmann, K., Milan, M., Benes, V., Ansorge, W. et al. (1998). Systematic gain-of-function genetics in Drosophila. *Development* **125,** 1049-57.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* **103,** 211-25.

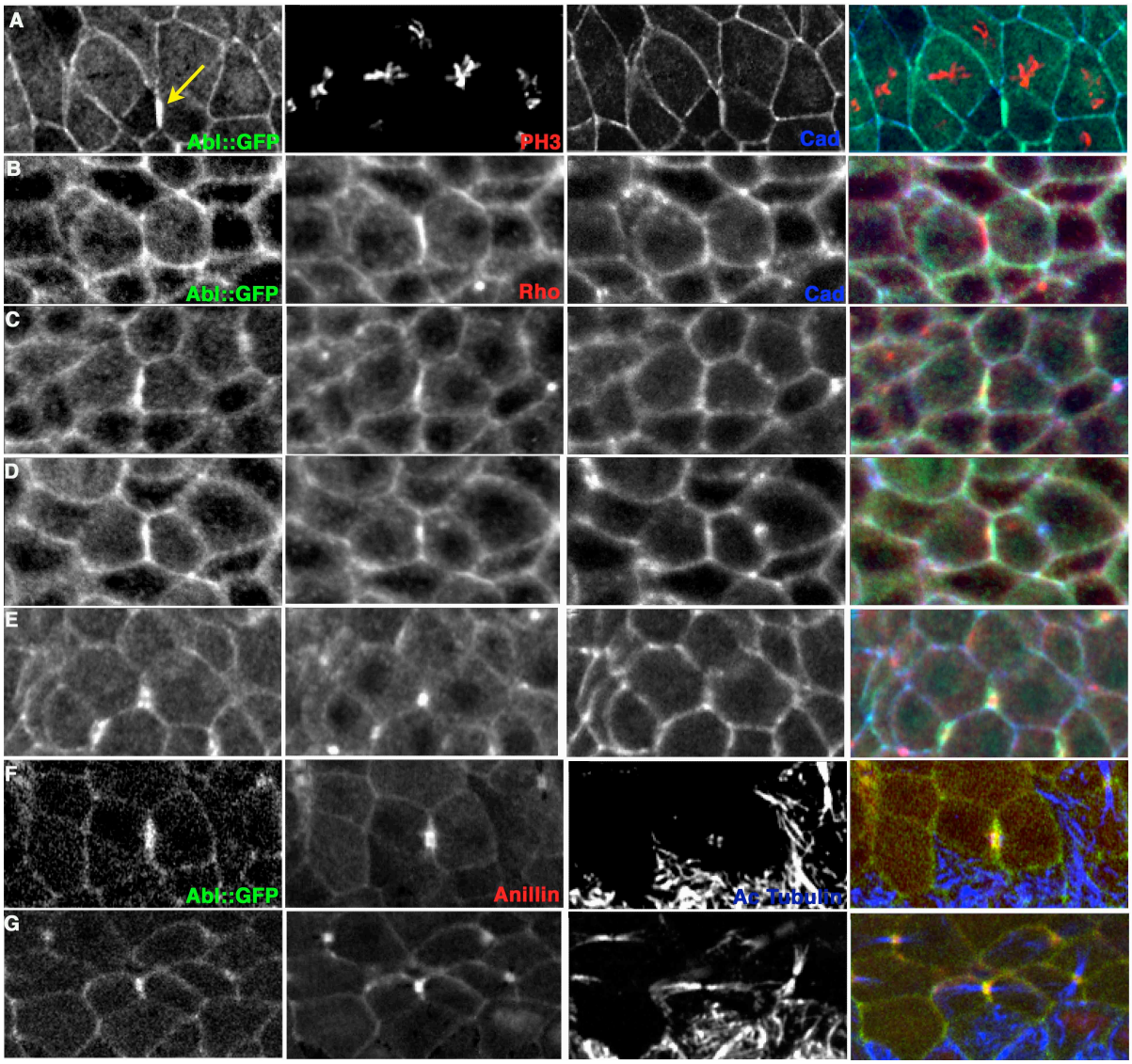
Somogyi, K. and Rorth, P. (2004). Cortactin modulates cell migration and ring canal morphogenesis during Drosophila oogenesis. *Mech Dev* **121,** 57-64.

Takahashi, M., Takahashi, F., Ui-Tei, K., Kojima, T. and Saigo, K. (2005). Requirements of genetic interactions between Src42A, armadillo and shotgun, a gene encoding E-cadherin, for normal development in Drosophila. *Development* **132,** 2547-59.

Tanis, K. Q., Veach, D., Duewel, H. S., Bornmann, W. G. and Koleske, A. J. (2003). Two distinct phosphorylation pathways have additive effects on Abl family kinase activation. *Mol Cell Biol* **23**, 3884-96.

Woodring, P. J., Hunter, T. and Wang, J. Y. (2003). Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. *J Cell Sci* **116**, 2613-26.

Fig. 5.1. Abl localizes to distinct structures late in mitosis. All images show mitotic domains in germband-extended embryos. A. Abl::GFP (green) is found at the apical cortex of all cells (arrow), but is enriched between non-Phospho-Histone H3 (red) positive cells of mitotic domains. Blue- DE-Cad marks cell apices. Note relative to Abl, DE-Cad is not as enriched at arrow. B. Mitotic domain, early telophase. Contractile rings contain Rho1 (red), but not Abl::GFP (green) or DE-Cad (blue). C. Mid-telophase. Abl::GFP (green) appears along the boundary between daughter cells, as Rho1 (red) begins to concentrate more at the center. D. Later in telophase than C. Abl::GFP (green) continues to be enriched at the boundary between daugther cells, while Rho1 (red) continues to coalesce into a more dot-like structure. E. Late telophase. Abl::GFP (green) now also coalesces in to a smaller structure than before (compare with B,C) and surrounds the dot-like Rho1 (red) structure. F. Co-localization of Abl::GFP (green) with the telophase markers Anillin (red) and Acetylated Tubulin (blue). At mid-telophase, Abl::GFP is enriched between daugther cells, and overlaps but also extends beyond the domain of anillin localization. Acetylated tubulin begins to appear perpendicular to the Abl/Anillin structures. G. Late telophase. Abl::GFP (green) overlaps anillin (red) and is bisected by a more extended domain of acetylated tubulin (blue).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Cad
B
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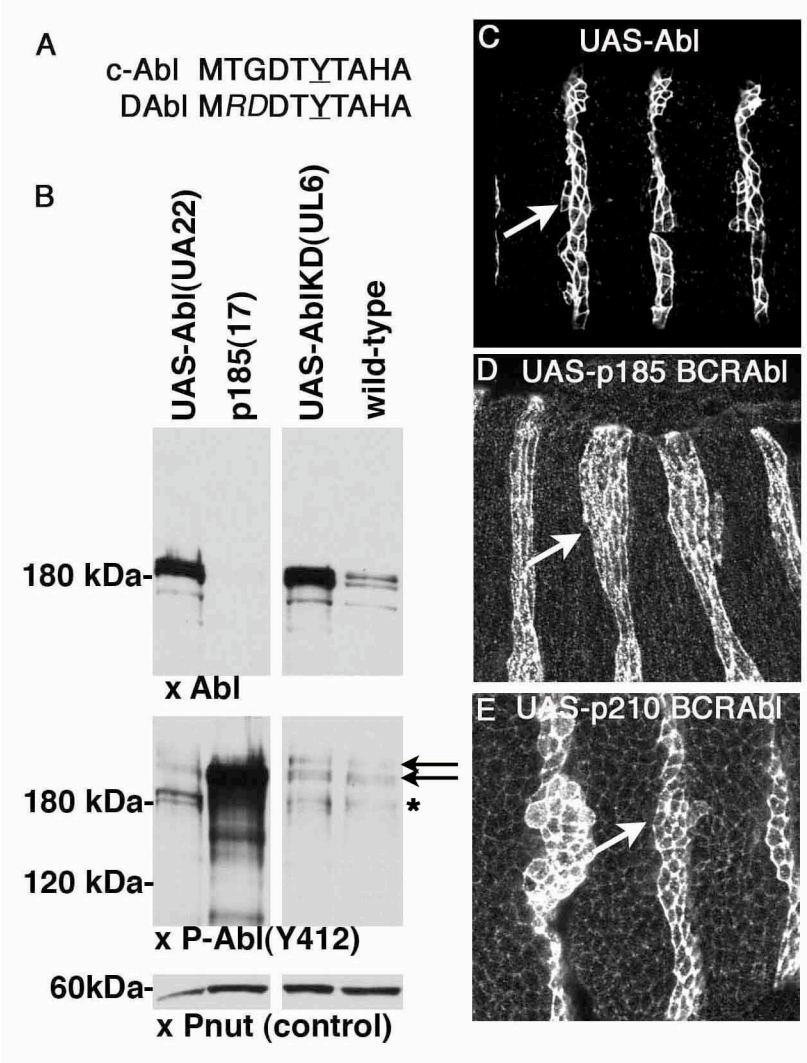
Rho
Cad
C
D
E
F
Abl::GFP
Anillin
Ac-Tubulin
G

Fig. 5.2. An antibody that recognizes activated *Drosophila* Abl. A. Alignment of the peptide sequence from c-Abl used to generate the P(Y412)Abl antibody with sequence from *Drosophila* Abl. Non-identical residues in fly are in italics. The phosphorylated tyrosine is underlined. B. Western blot from embryonic extracts. Upper panel- anti Abl blot. In UAS-Abl, UAS-Kinase dead Abl, and wild-type, anti-Abl recognizes a doublet at around 180 kDa. UAS-p185 BCR Abl is not recognized by this antibody due to the presence of mammalian sequence. Middle panel- anti P(Y412)Abl blot. Abl signal (marked with *, arrows mark cross-reacting bands) is elevated in embryos overexpressing wild-type or active (p185 BCR) Abl, but not in kinase dead Abl or wild-type embryos. Lower panel- anti Pnut (loading control). C. P(Y412)Abl recognizes UAS-Abl expressed in engrailed stripes. D. P(Y412)Abl recognizes UAS-p185BCRAbl expressed in engrailed stripes. E. P(Y412)Abl recognizes UAS-p210Abl expressed in engrailed stripes.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B
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p185(17)
UAS-AblKD(UL6)
wild-type
180 kDa-
x Abl
180 kDa-
120 kDa-
*
x P-Abl(Y412)
60kDa-
x Pnut (control)
C UAS-Abl
D UAS-p185 BCRAbl
E UAS-p210 BCRAbl

Fig. 5.3. Sub-cellular localization of Phospho-Abl during embryogenesis. Wild-type embryos, stained with P-Abl. A. Gastrualtion, ventral surface. P-Abl localizes to the apical cell cortex. B. Closeup of A showing tricellular junction localization (arrow). C. Germband extension, dorsal surface. P-Abl outlines both epithelial (lower half) and amnioserosal (wavy cells in upper half) cells. D. Germband extension, lateral epidermis, interior section. Arrow highlights apical localization of P-Abl in tracheal pits. E. Dorsal closure, dorsal surface. P-Abl localizes to the leading edge actin cable (arrow), as well as epithelial and amnioserosal cells. F. Closeup of E showing tricellular junction localization (arrow). G. After dorsal closure. P-Abl localizes to the epithelial cell cortex. H. After dorsal closure, P-Abl localizes to the axons of the central nervous system

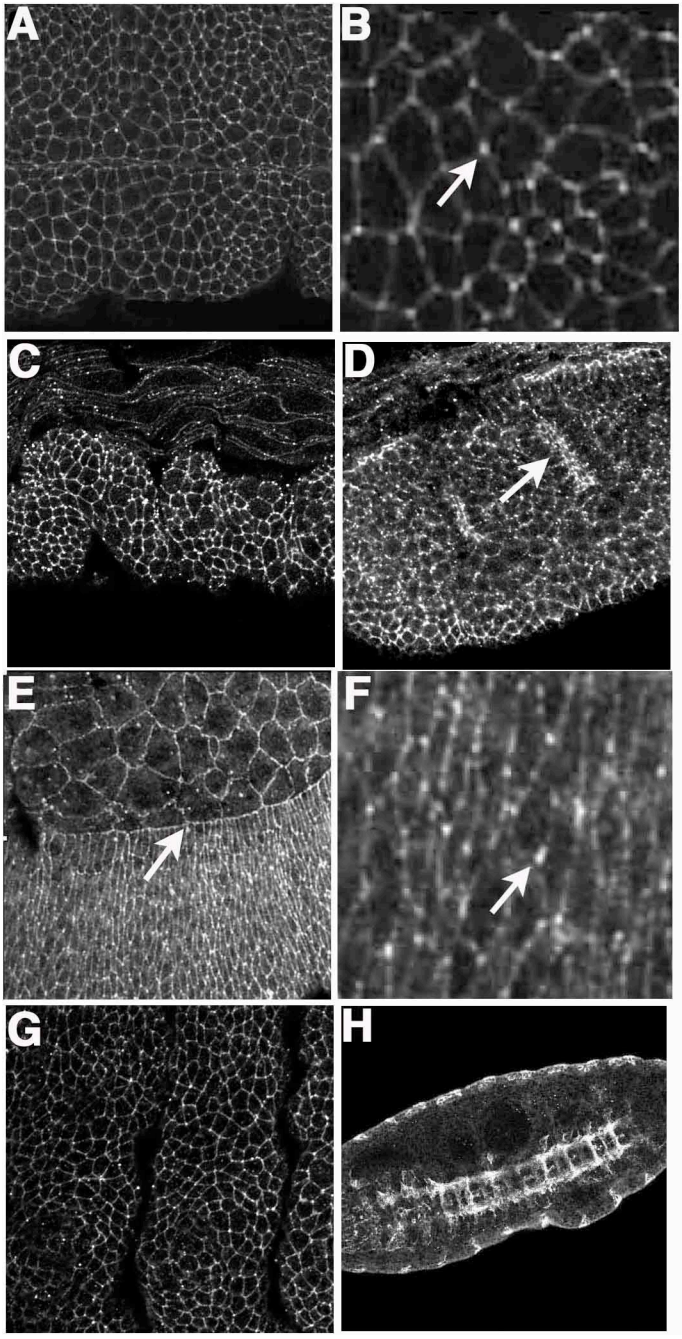
A
B
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Fig. 5.4. Co-localization of P-Abl with Abl::GFP and Ena. A. Cross-section, gastrulation, apical up. Abl::GFP (green) and P-Abl (red) localize prominently to the apices of epithelial cells (arrows). B. Lateral grazing section, germband extension. Abl::GFP (green) and P-Abl (red) are enriched between daugther cells (arrow) and at tri-cellular junctions (arrowhead). C. Lateral grazing section, germband extension. Ena (green) and P-Abl (red) co-localize at tri-cellular junctions. D. Dorsal grazing section, dorsal closure. Ena (green) is enriched in puncta at the leading edge (arrow) and in segmental groove cells (arrowhead). P-Abl (red) is not obviously enriched in either of these structures.

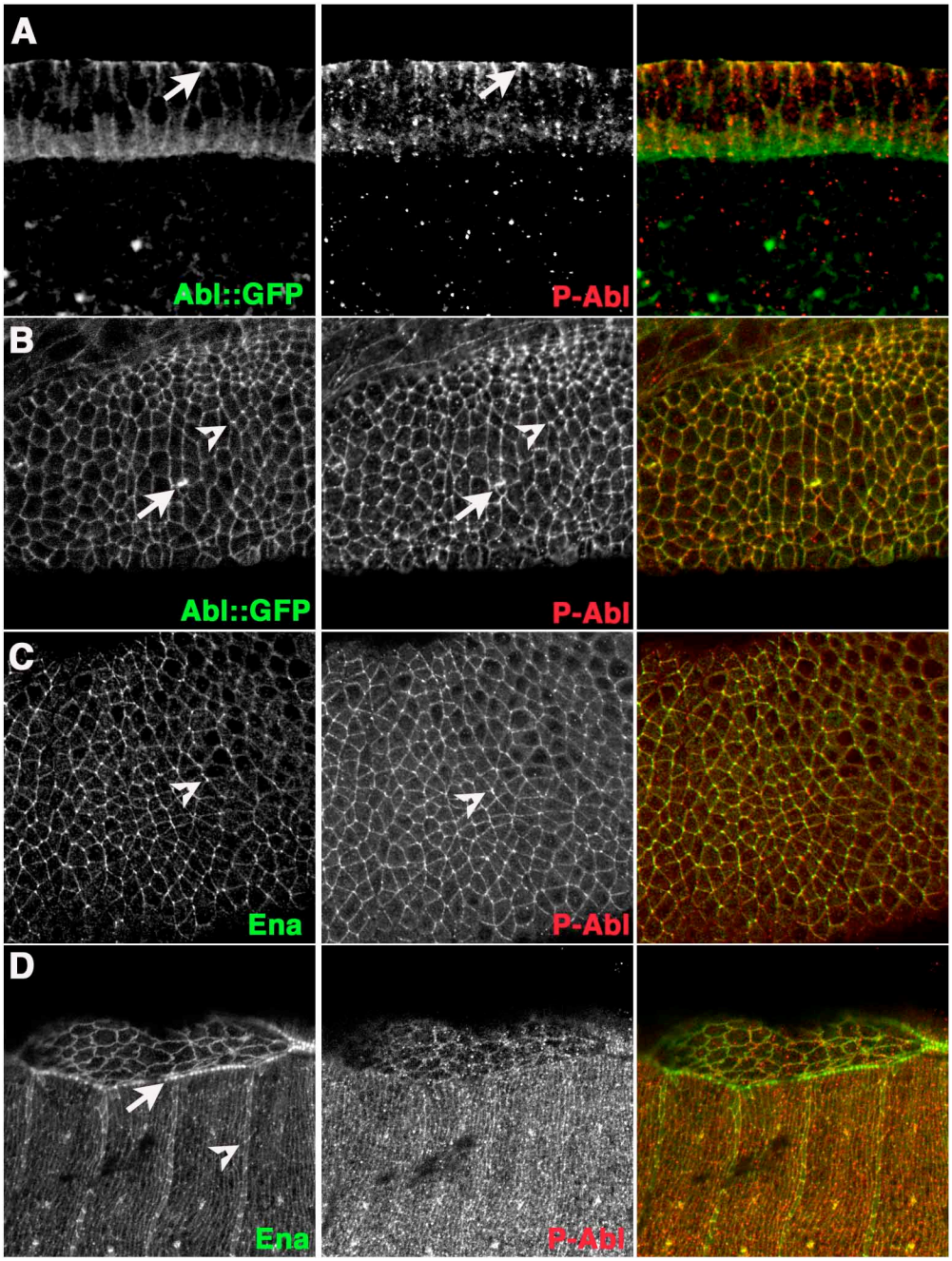
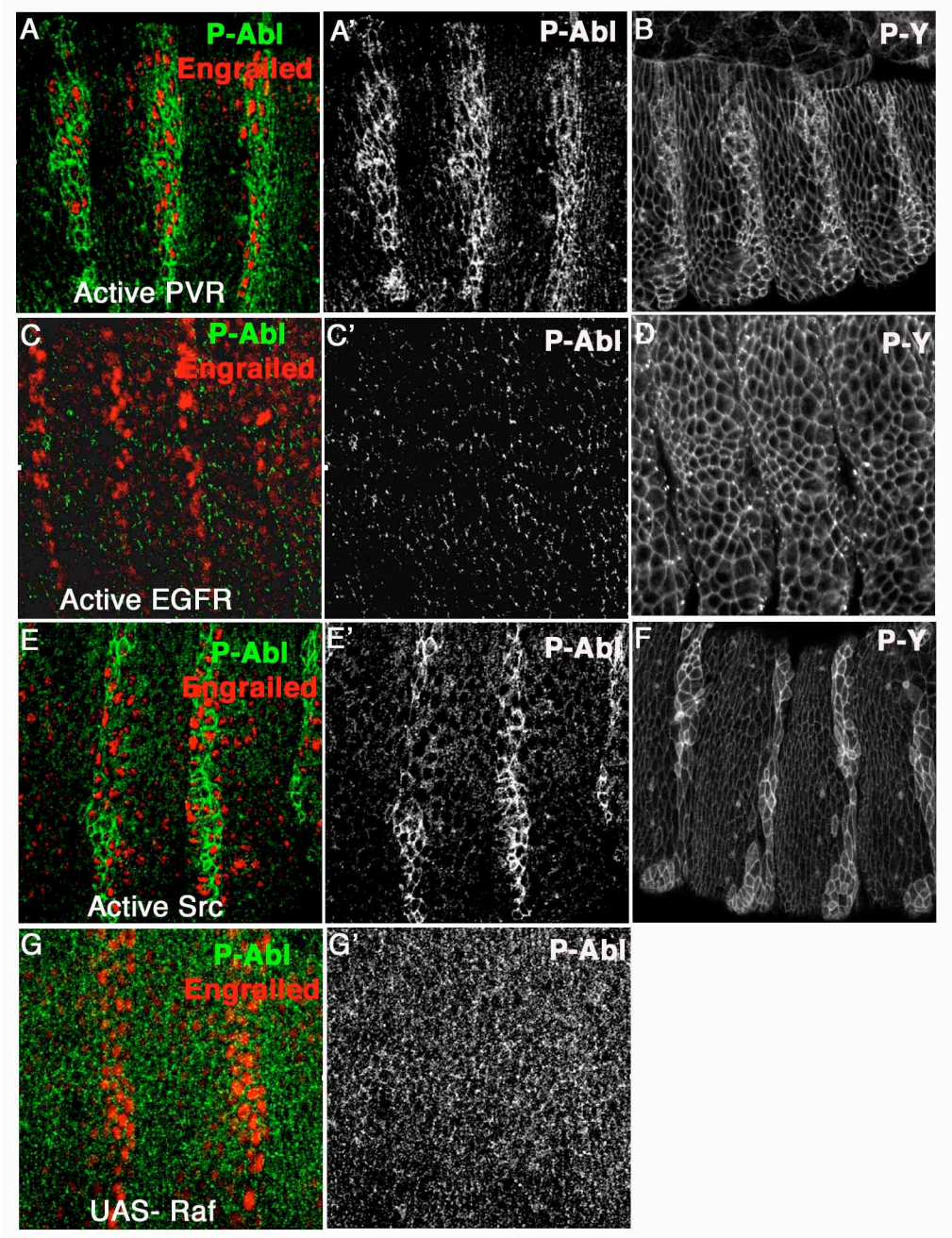

A
Abl::GFP
P-Abl
B
Abl::GFP
P-Abl
C
Ena
P-Abl
D
Ena
P-Abl

Fig. 5.5. PVR and Src42 are candidate Abl activators. All embryos express a given UAS construct under control of *engrailed* GAL4. In all merged images, red marks engrailed staining, and green shows P(Y412)Abl localization. A. Overexpression of PVR. A'. Abl activation is elevated in active PVR expressing cells. B. Phosphotyrosine (P-Y) increases in PVR expressing cells. C. Overexpression of EGFR. C'. Abl activation is not elevated in active EGFR expressing cells. D. Phosphotyrosine does not increase in EGFR expressing cells. E. Overexpression of Src42. E'. Abl activation increases in active Src expressing cells. F. Phosphotyrosine increases in Src42 expressing cells. G. Overexpression of Raf. G'. Abl activation does not increase in Raf overexpressing cells.


A
P-Abl
Engrailed
Active PVR
A'
P-Abl
B
P-Y
C
P-Abl
Engrailed
Active EGFR
C'
P-Abl
D
P-Y
E
P-Abl
Engrailed
Active Src
E'
P-Abl
F
P-Y
G
P-Abl
Engrailed
UAS- Raf
G'
P-Abl

CHAPTER 6

DISCUSSION

Building an animal is an intricate process on many levels. At the tissue level, layers are internalized, stretched, and compressed. Within these tissues, cells change shape or position via different mechanisms. At a molecular level, the complexity is greater still. Our current aim is to understand the interactions between the numerous signaling molecules that move the structural nuts and bolts of a cell. Doing so will illuminate the instructions that direct specific cell shape changes as part of overall tissue reorganization. Further, understanding the role of such morphogenesis regulators will contribute to our understanding of human disease. In my thesis work, I examined in detail the role of the signaling proteins Rho1 GTPase and Abl kinase during *Drosophila* epithelial morphogenesis. These proteins regulate specific aspects of cell shape change by interacting with adherens junctions and the actin cytoskeleton.

Rho GTPase: an integral part of morphogenesis

For the majority of my thesis work, the role of Rho1 GTPase was a recurring theme. Given the well-documented diversity of Rho GTPases in many cellular functions, it is not surprising that Rho1 contributes to multiple aspects of morphogenesis regulation. My work describes a role for Rho1 in adherens junction regulation, potentially through Cadherin trafficking. Further, Rho1 regulation is crucial during ventral furrow formation, downstream

of RhoGEF2. The challenge to understanding Rho1 function now is to tease a specific function away from another.

One key to separating the various roles for Rho GTPases is to focus on the roles of different Rho regulators and effectors. For example, the RhoGEF Pebble is a well-known regulator of Rho during *Drosophila* cytokinesis (reviewed in O'Keefe et al., 2001). Similarly, RhoGEF2 appears to participate in a variety of cell shape changes that involve apical cell constriction (Nikolaidou and Barrett, 2004). For Rho1's apparent role in adherens junction trafficking, a specific RhoGEF has yet to be identified. Doing so will further help to elucidate Rho's connection to adherens junction function.

Examining the targets of Rho will also tease apart Rho's various functions. In ventral furrow formation, Rho kinase appears to be a key target (Dawes-Hoang et al., 2005). Rho kinase mutants should be examined in the future to determine if this effector regulates both myosin accumulation and actin organization. Another potential Rho1 effector in this process could be the formin Diaphanous, known to regulate actin structure in *Drosophila* embryos (Afshar et al., 2000). If Rho1 regulates adherens junctions through trafficking, recent work in cultured cells suggests that Rho kinase may act downstream (Charrasse et al., 2006). Clearly, use of a variety of regulators and effectors is the key to specificity of Rho function for a given cellular process.

Abl kinase regulates specific aspects of apical morphogenesis

While Rho function appears quite ubiquitous, Abl kinase appears to act only in specific processes. My thesis work confirms through both phenotypic and localization studies that Abl acts primarily in the apical region of epithelial cells. Further, while Abl localizes apically in all epithelia, Abl is required for a subset of apical morphogenetic events.

This suggests that the localization of Abl activators/effectors may control Abl's input into a process. Two instances of such Abl-dependent processes are constriction of the mesoderm and elongation of the leading edge cells (Grevengoed et al., 2001). In both of these events, Abl's input into morphogenesis seems to be via regulation of actin structure. Actin has a unique localization in each of these tissues- dynamically relocalizing to the apical surface of constricting mesoderm and to the leading edge cable at dorsal closure. Future studies of these two processes and of Abl family proteins in other systems will advance our understanding of Abl's utility in the molecular toolkit that acts during morphogenesis.

One apparent key to Abl function is the regulation of Ena. As during dorsal closure and blastoderm stages, Ena is an Abl effector in ventral furrow formation. While Abl's regulation of Ena appears central to Abl function, the nature of the Abl/Ena interaction may be more complex than previously thought. During blastoderm development and in the mesoderm, Abl accumulates where Ena is not seen. In later epithelial cells, however, Abl and active Abl closely localize with Ena. This suggests that Abl's negative regulation of Ena may be more complex than previously thought- repelling Ena in one case while sequestering it in another. Future studies of the mechanism by which Abl and Ena interact will clarify the interaction of these proteins during cytoskeletal remodeling, including whether Abl kinase activity is necessary.

Other roles and partners for Abl

Additional roles and targets of *Drosophila* Abl during epithelial morphogenesis remain to be studied. For instance, some *abl* mutant embryos fail in germband retraction and dorsal closure. An interesting model for Abl function in these events could be as a downstream target of integrin-based adhesion. The evidence for this model in *Drosophila* is

twofold: Abl::GFP overlaps with Beta-integrin (Chapter 3) beginning at gastrulation and both germband retraction (Schock and Perrimon, 2002) and dorsal closure (Narasimha and Brown, 2004) require integrin function. Further, work in cultured mammalian cells points to a role for integrin engagement in Abl activation (Lewis et al., 1996), a function that could be explored in flies using the active Abl antibody.

Another interesting connection between Abl and morphogenesis could involve regulation of microtubules. Arg can cross-link actin and microtubules during fibroblast adhesion (Miller et al., 2004), and *Drosophila* Abl was recently shown to regulate the microtubule associated protein Orbit/MAST (Lee et al., 2004). My work on ventral furrow formation showed a failure of RhoGEF2-dependent myosin contraction in *abl* mutants. Given the model that RhoGEF2 is recruited to microtubule plus ends and stabilized in the apical cortex in constricting cells (Rogers et al., 2004), an attractive mechanistic model for Abl function in this process could be as an actin/microtubule cross-linker that indirectly stabilizes RhoGEF2 in the vicinity of apical actin.

Examining the role of candidate Abl activators will further clarify Abl's role in morphogenesis. Particular activators may act in different contexts. For instance, PVR is not expressed in early embryos (Rosin et al., 2004), where Abl clearly is functional. In contrast, Src42 localizes to many places in early embryos, including enrichment in the ventral furrow (Takahashi et al., 2005), suggesting that Src42 may direct the apical activation of Abl in the mesoderm. Of course, further study will clarify whether Pvr and Src42 are bona fide Abl activators.

Adherens junctions as hubs for signaling activity

Adherens junctions are critical for the maintenance of epithelial integrity. These cell adhesion contacts are remodeled during epithelial morphogenesis. Additionally, many proteins localize in the vicinity of adherens junctions to direct morphogenetic change. Our work in the *Drosophila* embryo supports this conclusion. For example, Abl and Ena localization overlaps adherens junctions in *Drosophila* epithelia. Both of these proteins participate in morphogenesis specifically in the apical domain of cells. Further, RhoGEF2 exhibits a dramatic relocalization to adherens junctions in apically constricting cells of the ventral furrow.

While many proteins may localize to adherens junctions, they may not directly act on junctions, but rather on the cytoskeleton. This is likely the case with Abl, which via Ena regulates actin. *abl* mutant ventral furrow phenotypes clearly effect actin and do not resemble those of adherens junction mutants (Dawes-Hoang et al., 2005). Another possibility is that a signaling protein acts on both adhesion and the cytoskeleton. For instance, Rho1 may regulate Cadherin trafficking but also actin structure at the leading edge during dorsal closure. Deciphering the primary targets/mechanisms of actions for other piece mover proteins is critical in the ongoing efforts to understand how signaling molecules direct morphogenesis.

Morphing forward

To date, many regulators of morphogenesis have been identified in several systems and countless processes. What is important now is to move from lists of genes that are tied to a process to regulatory networks, explaining how each piece of the puzzle fits together. It is important to identify who is in or out of a specific pathway as well as where different

pathways intersect. Studying related processes across evolution will further elucidate key signaling instructions that govern cell shape change. For example, our study of apical constriction in gastrulating *Drosophila* revealed that ventral furrow regulation consists of inputs into both actin and myosin regulation. While Abl and Cta contribute to only one of these inputs, RhoGEF2 cooperates in both. Further, Abl and RhoGEF2 appear to both regulate actin, but not as part of the same pathway. Our work points to conserved roles for Abl and Rho in actin regulation during ventral furrow formation. It will be interesting to see if the same is true for Abl and Rho during vertebrate neural tube closure.

Morphogenesis and disease

It is rewarding as a scientist to envision how discoveries at a basic research level may contribute to understanding human disease. The regulation of morphogenesis relates to human disease on many levels. Defects in morphogenesis can give rise to birth defects or lethality during pregnancy. Further, failure to properly regulate the cytoskeleton and adhesion is thought to be a primary step in the progression to cancer.

Neural tube closure defects are the second most common form of birth defect (reviewed in Detrait et al., 2005). While Folic Acid supplements have been effective in reducing the incidence of such defects, certain genetic backgrounds in mice appear resistant to this treatment (reviewed in Juriloff and Harris, 2000). My cross-sectional imaging of the ventral furrow reinforces the similarity between the internalization of these cells and those of the vertebrate neural tube. Given the conserved role for Abl in both of these processes and the existence of Abl-specific inhibitors, Abl could represent an important therapeutic target for prevention of neural tube defects.

Examining Abl's function also ties into understanding the abnormal activity of this kinase during chronic myeloid leukemia (CML). This condition is brought about by the Philadelphia chromosomal translocation that fuses part of the *bcr* gene to the extreme N-terminus of *abl* coding sequence. This fusion is thought to relieve auto-inhibitory interactions in Abl, resulting in an overactive kinase (reviewed in Hantschel and Superti-Furga, 2004). Our lab's work on Abl underscores the role of this kinase in regulation of actin structure via negative regulation of Ena/VASP. Studies in cultured mammalian cells show that in the absence of Ena/VASP, the actin network is more lamellipodial and turns over at a slower rate, leading to altered migration (reviewed in Sutherland and Way, 2002). As changes in migration rates are often a hallmark of cancer cells, this may suggest that inappropriate Ena/VASP regulation is important along the path to CML. Not surprisingly, cells from CML patients have been found to have an altered cytoskeleton and migration rate (Dong et al., 2003; Salgia et al., 1997).

Finally, improper regulation of adhesion at adherens junctions by Rho GTPases has also been linked to cancer. Epithelial tumors constitute the overwhelming majority of human cancers. Such tumors are characterized by the de-differentiation of polarized epithelial cells into invasive cells. Among the changes required to transform epithelial cells is a loss of cell-cell contact at adherens junctions (reviewed in Lozano et al., 2003). Our work and the work of others supports the idea that Rho GTPase is one of the key stabilizing forces in the maintenance of cell-cell contact. Thus, Rho GTPases may identify an important de-differentiating factor that could play a role in cancer therapy. An important theme for all of these studies is to first understand what a protein does when things go right (during normal development) before investigating what a protein does when things go wrong (in disease).

References

Afshar, K., Stuart, B. and Wasserman, S. A. (2000). Functional analysis of the Drosophila diaphanous FH protein in early embryonic development. *Development* **127**, 1887-97.

Charrasse, S., Comunale, F., Grumbach, Y., Poulat, F., Blangy, A. and Gauthier-

Rouviere, C. (2006). RhoA GTPase regulates M-cadherin activity and myoblast fusion. *Mol Biol Cell* **17**, 749-59.

Dawes-Hoang, R. E., Parmar, K. M., Christiansen, A. E., Phelps, C. B., Brand, A. H. and Wieschaus, E. F. (2005). folded gastrulation, cell shape change and the control of myosin localization. *Development* **132**, 4165-78.

Detrait, E. R., George, T. M., Etchevers, H. C., Gilbert, J. R., Vekemans, M. and Speer, M. C. (2005). Human neural tube defects: developmental biology, epidemiology, and genetics. *Neurotoxicol Teratol* **27**, 515-24.

Dong, R., Cwynarski, K., Entwistle, A., Marelli-Berg, F., Dazzi, F., Simpson, E., Goldman, J. M., Melo, J. V., Lechler, R. I., Bellantuono, I. et al. (2003). Dendritic cells from CML patients have altered actin organization, reduced antigen processing, and impaired migration. *Blood* **101**, 3560-7.

Grevengoed, E. E., Loureiro, J. J., Jesse, T. L. and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in Drosophila. *J Cell Biol* **155**, 1185-98.

Hantschel, O. and Superti-Furga, G. (2004). Regulation of the c-Abl and Bcr-Abl tyrosine kinases. *Nat Rev Mol Cell Biol* **5**, 33-44.

Juriloff, D. M. and Harris, M. J. (2000). Mouse models for neural tube closure defects. *Hum Mol Genet* **9**, 993-1000.

Lee, H., Engel, U., Rusch, J., Scherrer, S., Sheard, K. and Van Vactor, D. (2004). The microtubule plus end tracking protein Orbit/MAST/CLASP acts downstream of the tyrosine kinase Abl in mediating axon guidance. *Neuron* **42**, 913-26.

Lewis, J. M., Baskaran, R., Taagepera, S., Schwartz, M. A. and Wang, J. Y. (1996). Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport. *Proc Natl Acad Sci U S A* **93**, 15174-9.

Lozano, E., Betson, M. and Braga, V. M. (2003). Tumor progression: Small GTPases and loss of cell-cell adhesion. *Bioessays* **25**, 452-63.

Miller, A. L., Wang, Y., Mooseker, M. S. and Koleske, A. J. (2004). The Abl-related gene (Arg) requires its F-actin-microtubule cross-linking activity to regulate lamellipodial dynamics during fibroblast adhesion. *J Cell Biol* **165**, 407-19.

Narasimha, M. and Brown, N. H. (2004). Novel functions for integrins in epithelial morphogenesis. *Curr Biol* **14**, 381-5.

Nikolaidou, K. K. and Barrett, K. (2004). A Rho GTPase signaling pathway is used reiteratively in epithelial folding and potentially selects the outcome of Rho activation. *Curr Biol* **14**, 1822-6.

O'Keefe, L., Somers, W. G., Harley, A. and Saint, R. (2001). The pebble GTP exchange factor and the control of cytokinesis. *Cell Struct Funct* **26**, 619-26.

Rogers, S. L., Wiedemann, U., Hacker, U., Turck, C. and Vale, R. D. (2004). Drosophila RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner. *Curr Biol* **14**, 1827-33.

Rosin, D., Schejter, E., Volk, T. and Shilo, B. Z. (2004). Apical accumulation of the Drosophila PDGF/VEGF receptor ligands provides a mechanism for triggering localized actin polymerization. *Development* **131**, 1939-48.

Salgia, R., Li, J. L., Ewaniuk, D. S., Pear, W., Pisick, E., Burky, S. A., Ernst, T., Sattler, M., Chen, L. B. and Griffin, J. D. (1997). BCR/ABL induces multiple abnormalities of cytoskeletal function. *J Clin Invest* **100**, 46-57.

Schock, F. and Perrimon, N. (2002). Cellular processes associated with germ band retraction in Drosophila. *Dev Biol* **248**, 29-39.

Sutherland, J. D. and Way, M. (2002). Looking over the edge: a new role for Ena/VASP proteins in lamellipodial dynamics. *Dev Cell* **2**, 692-4.

Takahashi, M., Takahashi, F., Ui-Tei, K., Kojima, T. and Saigo, K. (2005). Requirements of genetic interactions between Src42A, armadillo and shotgun, a gene encoding E-cadherin, for normal development in Drosophila. *Development* **132**, 2547-59.